IMPLANTABLE ALGINATE MICROCAPSULES AS GENE THERAPY FOR HEMOPHILIA A

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By

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ABSTRACT

Hemophilia A is an X-linked recessive bleeding disorder caused by the deficiency of coagulation factor VIII [1]. Current treatment for hemophilia A consists of prophylactic or on demand replacement therapy of either plasma-derived or recombinant FVIII concentrates [2]. Albeit effective, there are several limitations associated with factor concentrates, including high cost that limits its availability for close to 80% of hemophilia patients in developing countries [3-5]. An alternative treatment would thus be desirable. Gene therapy for hemophilia has seen many successes in animal models and represents a more cost-effective alternative to the current treatment modalities [6]. In the current work, I present a gene therapy system for hemophilia that uses mouse fetal myoblasts engineered to secrete FVIII, enclosed in immuno-protective alginate-poly-L-lysine-alginate (APA) microcapsules, as a sustained source of FVIII. In this study, a thorough examination of the encapsulated myoblasts using a novel flow cytometry assay was performed. This method yielded an accurate and precise method for encapsulated cell viability calculation, and also allowed for analysis of several other parameters such as health (cell morphology), cell size and distribution. Flow cytometry was also used to monitor the time-course proliferation profile of encapsulated myoblasts secreting cFVIII, using the division tracking dye CFSE. We found that encapsulated cells display a decreased proliferation rate as well as lower viability than non-encapsulated cells. Implantation of encapsulated G8 myoblasts secreting cFVIII into hemophilia A mice resulted in maximum plasma levels of protein on day 1 (~18% of normal canine FVIII

levels). Delivery of cFVIII in hemophilic mice also offered protection against blood loss after the mice were subjected to injury (as measured by hematocrit levels); indicating that biologically functional cFVIII continued for at least 7 days post-capsule implantation. Low levels of FVIII antigen returned on day 28 after a transient disappearance on day 14. However, the presence of antigen must be reconciled with appearance of anti-cFVIII antibodies that were detected in the plasma of treated mice at the end of five weeks. The neutralizing nature of these antibodies still needs to be characterized by Bethesda assay Overall, our study demonstrates the feasibility of delivering therapeutic levels of FVIII using encapsulated G8 fetal myoblasts. The presence of functional FVIII protein on day 7, suggests that this treatment was not met by transcriptional repression *in vivo,* thereby overcoming one of the major obstacles faced by using the transformed C2C12 cell line secreting hFVIII [7] If such levels of FVIII were achieved in humans, it would be sufficient to convert a severe hemophiliac into a mild phenotype. Thus, this gene therapy strategy may be a suitable therapeutic alternative for hemophilia patients. Further work ought to focus on the long-term persistence of FVIII in hemophilia A mice, and also determining the protection following trauma over time to determine if the FVIII remains functional. Other cell lines should be explored for higher expression, reduced immunogenicity and improved viability Still, there is a need to develop human cells expressing high levels of biologically active hFVIII with similar properties to the fetal cells described in this study [8, 9]

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If you, the reader are taking the time to read this thesis beyond the acknowledgements page, then you have already surpassed all of my expectations.

Lastly, as a gift for his retirement from his own accomplished career as a scientist, I would like to dedicate this thesis to my dad, who has always taught me that with hard work and big ideas you can achieve greatness.

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1. LITERATURE REVIEW

1.1 Hemophilia

Bleeding results when a capillary or a larger vessel is damaged. Hemostasis is the body's protective physiological response to vessel injury by the rapid formation of a blood clot, comprised of both platelets and fibrin [10, 11]. The injury results in the exposure of blood components to the sub-endothelial layers of the vessel wall, which initiate the hemostatic process. The interaction of platelets at the site of vessel injury as well as several coagulation factors result in fibrin deposition and the formation of a stable hemostatic plug. There are certain individuals who suffer from hemophilia, a disease caused by deficiencies in specific blood proteins critical in the coagulation process. In particular, the two common types of hemophilia that exist, hemophilia A and B, are caused by reduced levels or the absence of clotting factor FVIII and clotting factor IX, respectively [4]. Since the genes for both these proteins are located on the X-chromosome, hemophilia follows an X-linked pattern of inheritance. As a result, virtually all hemophiliacs are males (Figure 1)

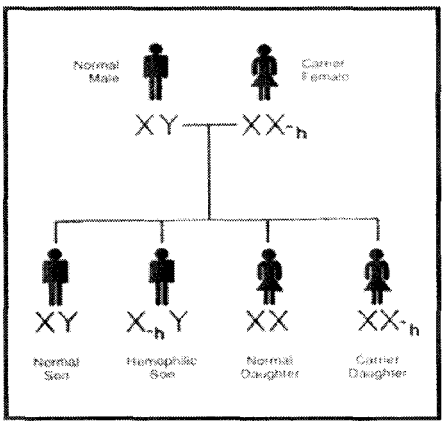


Figure 1 Genetic Pattern of Inheritance for Hemophilia. Mutations in the FVIII (Xq28) or FIX (Xq27.1-q27.2) gene on the X-chromosome, may lead to expression of the disease phenotype in affected males. Since only females who inherit mutations in both X-chromosomes present with the disease, they are often carriers. Canadian Hemophilic Society (2006).

Clinically, hemophilias A and B are indistinguishable from each other, and can only be diagnosed with a specific factor assay to determine the missing clotting factor [12].

1.1.1. Hemophilia A

The prevalence of hemophilia A is approximately five times that of hemophilia B, affecting 1 of every 5000 males [13]. Many of those affected only become aware of their condition at the first presentation of bleeding, which may be experienced anywhere from infancy to much later on in life depending on the severity of the disease, which is generally correlated with the level of coagulation protein in their blood. Three distinct phenotypes are typically recognized: severe, moderate or mild.

Table 1 Classification of Hemophilia A. The severity of the bleeding can be predicted by the plasma level of FVIII found in the patient. Normal FVIII concentration in human plasma = 100-200 ng/ml

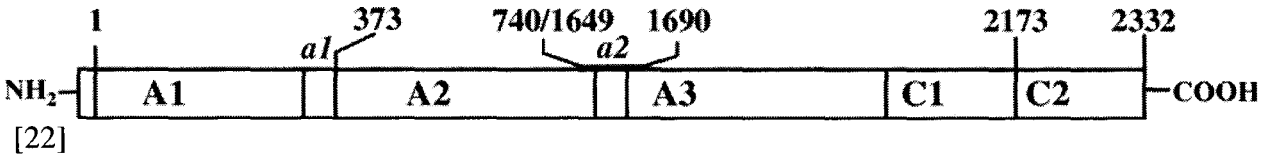
Classification	FVIII Activity	Clinical Characteristics
Severe	<1% of normal	Spontaneous joint and muscle bleeding
Moderate	1-5% of normal	Prolonged bleeding after minor injury
Mild	5-40% of normal	Mild episodes of bleeding, usually discovered after severe trauma.

The clinical classifications of hemophilia can help predict the bleeding risk of the patient, along with the management strategy and frequency of treatment they require. Spontaneous bleeding into muscle and joints (acute hemarthrosis) is the clinical hallmark for severe hemophilia, and may have destructive and painful consequences if inadequately treated. These continuous bleeds can lead to irreversible arthropathy, joint deformation (requiring joint replacement), subsequent disability, and ultimately a loss of full participation in normal activities [14]. Compared to the normal physiological level of 100-200 ng/ml, severe hemophiliacs carry less than 1% of normal FVIII levels in their blood and may require frequent factor VIII replacement therapy several times a month [15]. Most children with severe hemophilia will experience their first bleed into a joint by age 2, but many others will bleed from other sites at an earlier age. Moderate hemophilia, characterized by FVIII levels between 1-5% of normal, is usually diagnosed

by the age of 5 years, and may lead to spontaneous bleeding, but less frequently than patients with the severe condition [16]. Mild hemophiliacs rarely suffer from unprovoked episodes of bleeding, and may only be detected much later on in life because of prolonged post-operative bleeding. It becomes obvious then, that the minimal objective in the treatment for hemophilia is to deliver a sufficient amount of FVIII protein to convert the severe and moderate hemophiliac into a milder phenotype so that they may live a relatively normal life. Since FVIII is the major player when discussing hemophilia therapies, it is important to understand both its gene and protein structure when designing different strategies for its delivery into patients.

1.2. Factor VIII

FVIII is a glycoprotein in the blood that plays a critical role in blood coagulation, and is the clotting factor that is reduced or absent in haemophilia A patients. The primary structure of human FVIII was first deduced based on its DNA sequence in 1984 [17, 18]. The site of factor VIII synthesis has remained unclear for a long time. Although FVIII mRNA has been found in many other cells and tissues, the most likely location is in the hepatocytes, [19, 20]. After processing of the signal peptide, the mature FVIII molecule is translated as a single polypeptide chain with a molecular weight of about 300 kDa. The FVIII sequence is divided into three A, two C, and a large B domain(s), abbreviated as (NH_2)-A1-A2-B-A3-C1-C2-(COOH) [17, 21].



[22]

Figure 2 The protein structure of FVIII. There are 2332 residues in the mature FVIII molecule comprised of 3 distinct domain types. The large B-domain (not shown) resides between the A2 and A3 domains [22]

The secreted protein does not remain as a single chain, but instead is secreted as a divalent metal ion-dependent heterodimer comprised of a 100 – 200 kDa variable-length heavy chain (A1-A2-B) and an 80kDa light chain (A3-C1-C2) [21]. Further still, the activated form, FVIIIa, circulates as a heterotrimer - A1/ A2/ A3-C1-C2 – as a result of enzymatic cleavage by thrombin or factor Xa [22]

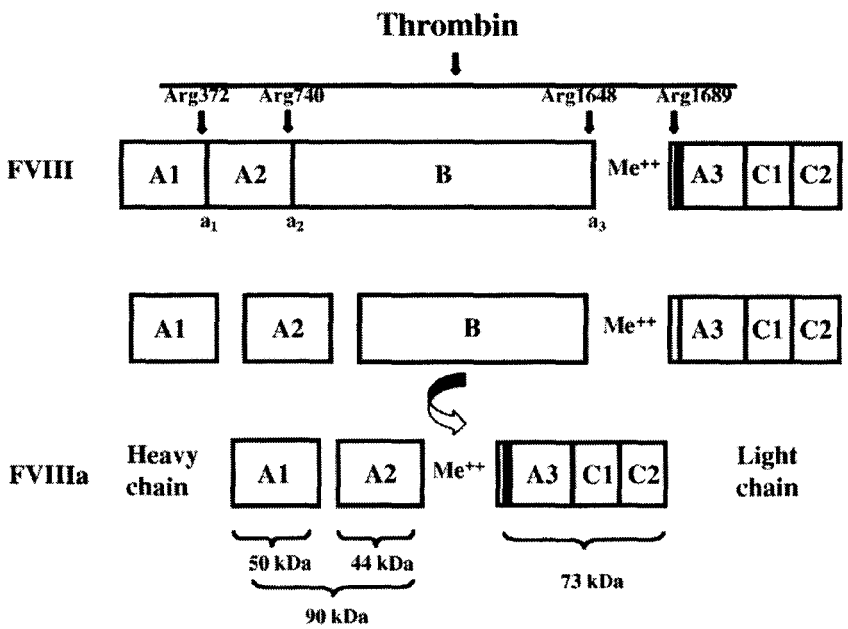


Figure 3 Activation of FVIII. FVIII circulates as a heterogeneous mixture of heterodimers formed by variable intracellular processing of the B domain, complexed with its carrier protein von Willebrand factor (vWF). The diagram shows the expected size fragments because of thrombin cleavage at Arg372, Arg740, and Arg1689. FVIIIa is formed by thrombin cleavage at residues A1—A2 junction and A2—B junction in the heavy chain, and residue 1689 in the light chain of FVIII [22]

The activity of FVIII is not controlled by the central B domain, allowing most of the large central B domain (>700aa) to be deleted without loss of FVIII coagulant function. [23, 24]. As a result, the B-domain deleted (BDD) form of FVIII is often used for cloning into vectors (for the production of recombinant FVIII), due to the significant reduction in size.

The mature form of FVIII circulates in the plasma at approximately 100-200 ng/ml. However, it does not circulate alone. When secreted, FVIII is closely associates with a protein called von Willebrand factor (vWF) which helps to stabilize FVIII by minimizing its proteolysis, as well as helps target and concentrate the clotting factor to the vascular wall upon injury. Prior to FVIII activation, vWF remains bound with high affinity (K_d ~0.4nm) to FVIII's B and A3 domain (N-terminal) and prevents premature formation of the activation complex [25].

1.2.1. FVIII and the Coagulation Process

FVIII is activated at the site of the coagulation event and is one of many proteins that participate in a series of complex proteolytic events (that lead to clot formation), well known as the coagulation scheme. FVIII and the rest of the coagulation factors respond to injured blood vessels to form fibrin strands, which functions to strengthen the initial cellular platelet plug formed during primary haemostasis [26]. The coagulation scheme has two pathways that lead to fibrin formation: the contact activation pathway (formerly known as the intrinsic pathway) and the tissue factor pathway (formerly known as the extrinsic pathway). The pathways are made up of a series of reactions, in which zymogens (inactive enzyme precursors) are activated to catalyze the next reaction in the cascade, ultimately resulting in cross-linked fibrin [27] Most of the coagulation factors (with the exception of two glycoprotein's FVIII and FV), are zymogens that are activated into serine proteases: a class of enzymes that have a serine residue at the catalytic centre and cleave peptide bonds in proteins after lysine or arginine residues. FVIII and FV act as co-factors to FIX and FX respectively. Coagulation factors are generally indicated by Roman numerals, with a lowercase "a" to represent the activated form.

1.3. Clotting Process

The main role of the tissue factor pathway is to generate the initial burst of thrombin. As the penultimate enzyme in the generation of cross-linked fibrin, and its role in feedback activation of FV, FVIII and FXI, thrombin is considered to be one of the most important proteins in the coagulation network. It is generally accepted that coagulation is

initiated by tissue factor (TF) *in vivo* [10]. Tissue factor is a protein present in the sub-endothelial tissue that enables cells to initiate blood coagulation. Unlike some of the other co-factors which circulate as non-functional precursors, TF is a potent initiator that is fully functional when expressed on the surface of cells [28]. The initiation step is thus localized to the cells that express TF, which are normally found outside the vasculature.

1.3.1. Initiation

Upon damage to the blood vessel, sub-endothelial TF is exposed to blood forming a complex with factor VII/FVIIa. This activated FVIIa/TF complex activates small amounts of factors IX and X. This factor Xa associates with the sub-endothelium or platelets where it generates sub-nanomolar thrombin. This sub-nanomolar thrombin activates FV, cofactor to FXa, to form a prothrombinase complex on the surface of the tissue-factor bearing cell, which more efficiently converts prothrombin to thrombin. Almost immediately, tissue factor pathway inhibitor (TFPI) inhibits FXa, and FXa – TFPI in turn inactivates FVIIa/TF. This action of TFPI localizes coagulation to the TF-bearing cell surfaces on which FVIIa/TF was formed [10, 29].

1.3.2. Amplification and Propagation

The small amount of thrombin that was already generated via Xa is sufficient to activate other components of the coagulation process, including FV, FVIII and FXI. Thrombin dissociates FVIII from the complex with vWF through specific proteolytic cleavages in both the heavy and light chains (Figure 3) [30, 31]. Free from vWF, activated FVIIIa can now form a "tenase" complex with FIXa that assembles on a

phospholipid membrane to catalyze the conversion of factor X to factor Xa in the presence of Ca^{2+}, thereby continuing the cycle [32].

FVIII is an essential co-factor of the intrinsic pathway, as it enhances FIXa catalysis by several orders of magnitude (~200,000-fold) [33-35]. Thus, normal functioning of FVIII requires that it interact with physiologically important ligands, namely vWF, phospholipids, FIXa, FX, FXa and thrombin. Molecular defects in FVIII, such as the mutations that lead to hemophilia A, may affect one or more of these critical interactions and result in a slowing down of the entire clotting process [36].

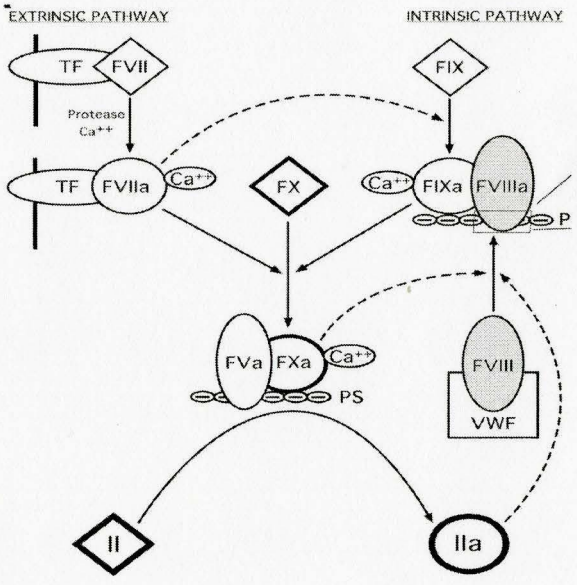


Figure 4 Role of FVIII in the coagulation process. FVIII glycoprotein acts as a regulator of the intrinsic pathway. Upon activation by thrombin, FVIIIa dissociates from vWF and associates with FIXa to further propagate FXa and thrombin generation [33].

1.4. Current Treatment for Hemophilia

There is currently no cure for Hemophilia A. The main treatment is to raise the concentration of FVIII in the blood to an adequate level such that spontaneous and traumatic bleeds can be prevented. Thus, current treatment for patients with hemophilia includes either plasma-derived FVIII or recombinant FVIII concentrates. Both treatments can be given as bleeds occur (on demand) or regularly to prevent bleeds before they occur (prophylaxis). Most severe hemophiliacs are on therapy in their own homes with intravenous infusions of the protein. The development of self-infusion and home therapy programmes became possible after plasma-derived FVIII and FIX concentrates became available in the 1970s [4]. The life expectancy of severe hemophiliacs has dramatically improved with the availability of FVIII and FIX concentrates for prophylactic treatment. In Sweden, for example, where the median life expectancy was once 20 to 25 years of age, it is now about 70 years at the beginning of the 21st century [37, 38]. Safety, cost and availability are the major determining factors when deciding on a replacement therapy [12].

1.4.1. Plasma-derived FVIII

Clotting factor concentrates purified from pooled plasmas of healthy individuals are commonly used to treat hemophiliacs. These plasma-derived clotting factors are much safer now than the products available prior to the 1990s, when there was a high risk for transmission of blood borne viruses associated with the use of plasma-derived FVIII concentrates. In the 1980s, up to 50% of hemophilia A patients were diagnosed as being

infected with human immunodeficiency virus (HIV) [39]. Hepatitis B transmission had been recognized in the past as complications of plasma-derived concentrates made from large pools of plasma (>20, 000 plasma donations). The safety of plasma-derived factors depends on the viral load in the plasma concentrate and the degree of inactivation of these viruses. Manufacturers of plasma-derived concentrates now employ at least two viral inactivation procedures. As a result, enveloped viruses, like hepatitis B or C and HIV, are no longer transmitted by plasma-derived concentrates [40, 41]. While plasma-derived FVIII products currently licensed for clinical use do not transmit blood borne pathogens and are considered safe and effective, the new recombinant FVIII products, which are free from any human or animal proteins, are similarly subjected to pathogen reduction strategies and also do not pose a risk for infection by known viruses or prions (abnormally structured proteins) [4, 12, 15].

1.4.2. Recombinant FVIII

The isolation of the FVIII gene in 1984 resulted in the rapid development and expression of recombinant FVIII in mammalian cells. Two preparations of full-length recombinant factor VIII were licensed for therapy of hemophilia A in the early 1990s. Both products showed excellent efficacy, with dose-dependent increases in the levels of factor VIII recovered in the plasmas of treated hemophiliacs [42-45]. Although attempts aimed at the generation of new more therapeutically-promising recombinant FVIII, these constructs always face the same pressures of extremely high costs in the developmental and manufacturing process. FVIII production in non-human mammalian cell lines comes

with many inherent disadvantages. They secrete low FVIII levels (relative to human liver the normal site of most FVIII synthesis), because of differences in the intracellular protein translation and posttranslational modification pathways, which could affect the biological activity of FVIII. Also, FVIII synthesis from nonhuman cell lines runs the risk of contamination with cellular components which may induce antigenic reactions. The production of human FVIII in a human cell line may overcome these disadvantages. For example, in comparison to human FVIII production in Chinese hamster ovary (CHO) cells and baby hamster kidney (BHK) cells, BDDrFVIII produced in a human embryonic kidney (HEK) cell line displays distinct differences in the N-glycan composition of the resulting FVIII. Thus its N-glycosylation pattern may be an important factor in determining the stability, efficacy and safety (i.e. immunogenicity) of FVIII products since these differences in N-glycosylation are detected by specific cell receptors [46] .

1.4.3. Inhibitors to FVIII

One of the most serious complications of FVIII replacement therapy is the development of inhibitory antibodies in about 25% of hemophilia A patients [43, 47]. Inhibitors inactivate FVIII function and decrease the effectiveness of the treatment so that bleeding episodes are no longer responsive to infusions of FVIII.

Inhibitor risk is associated with the type of mutation present in that patient. Patients with mutations of FVIII that severely reduce or prevent its production (large deletions, non-sense mutations) have a much higher frequency of inhibitor formation, indicating that a major driving force for inhibitor development is the presentation of a

novel antigen (the therapeutic FVIII), to the patient's immune systems of these patients [4, 22]. Up to 50% of individuals with severe hemophilia A but only 3-13% of those with mild or moderate hemophilia A develop inhibitors to replacement factor concentrate [48, 49]. The Bethesda and modified Bethesda assays are standard procedures for the detection and characterization of FVIII inhibitors and work by testing the ability of the patients' plasma to reduce FVIII activity in normal plasmas [50]. Patients with FVIII inhibitors are treated with higher and more frequent doses of human FVIII which leads to an even higher treatment cost. The goal of immune tolerance therapy is to eliminate inhibitors; however, prevention of their formation still remains an unresolved problem for FVIII replacement therapy.

1.4.4. Mutations in FVIII

Since the FVIII gene sequence was published in 1984, a large number of mutations that cause hemophilia A have been identified [51]. The only common FVIII defects which occur in 2% and 45% of severe hemophiliacs, are those of the intron 1 and intron 22 inversions, respectively [52, 53]. In the remaining severe hemophilic patients various types of mutations have been detected including nonsense, missense and splice-site mutations, as well as small or large insertions/deletions. Moderate or mild cases of hemophilia usually arise from missense mutations [54]. The emergence of inhibitors, antibodies that neutralize the pro-coagulant activity of FVIII, is associated with the type of mutation present in the FVIII molecule (along with other factors including the severity of the deficiency, patient ethnicity and treatment regimen) [55-58]. Patients with large

deletions, nonsense mutations and intron 22 inversions, for example, have a reported incidence of inhibitors to be higher than 30%. Also, nonsense mutations occurring in the second half of the FVIII gene are known to increase the risk of inhibitor formation [59]. A recent study of gene mutations in patients with hemophilia A revealed 26 novel FVIII mutations and four novel mutations that were associated with inhibitor development.

Although current management for hemophilia by intravenous infusion of either plasma-derived or recombinant FVIII is highly effective, it falls short of offering a long-lasting expression of the desired gene. In other words, patients are unable to maintain hemostasis without significant life-long medical intervention. Moreover, the cost of concentrate is approximately $100, 000 per year for an adult patient with a severe form of the disease – a cost that cannot be afforded by hemophiliacs in developing countries. Thus, a more economically feasible strategy is needed to by-pass the disadvantages of current treatments. Most patients would consider improvements to include products that require less frequent infusions, a non-intravenous route of administration, less patient involvement to reduce the risk of user error, and a method of therapy that would be less expensive than the factor products on the market today. Gene therapy represents an attractive alternative to the correction of genetic diseases, especially for hemophilia [2].

1.5. Gene Therapy

Gene therapy is a strategy involving the insertion of genes into an individual's cells and tissues for the treatment of inherited diseases, where a functional gene is delivered to replace the defective or mutated version. Generally, it is not an exact copy replacement of the abnormal disease-causing gene, but rather extra wild-type copies are provided to complement the loss of function. Gene therapy strategies can be grouped into two broad categories: *Ex vivo* and *in vivo* gene therapy.

Ex vivo gene therapy involves the *in vitro* genetic modification of cells harvested from a patient. Once the cells have successfully been genetically engineered to secrete the therapeutic protein of interest, they can be re-implanted into the same patient or animal [60]. In contrast, *in vivo* gene therapy involves the direct administration of the gene of interest into the patient, via some form of expression vector. The expression vectors used to deliver the transgene in both forms of gene therapy can either be of viral or non-viral origin.

Since viruses have established a method for effectively introducing their genetic material into host cells, they could also be manipulated and used in gene therapy to carry therapeutic genes into appropriate human cells. The disease-causing genes in the virus are removed and replaced with the genes encoding the protein missing or defective in the patient. Examples of viruses that can be used in this manner include retrovirus (RV), adenovirus (AV), adeno-associated virus (AAV), lentivirus (LV) and herpes simplex virus (HSV).

Gene delivery using non-viral approaches can be divided into either a physical (carrier-free gene delivery) or chemical (synthetic vector-based gene delivery) category. Physical approaches, including needle injection, electroporation, gene gun, ultrasound, and hydrodynamic delivery, utilize a physical force that will permeate the cell membrane to increase the efficiency of gene transfer. The chemical approaches employ synthetic or naturally occurring compounds as carriers to deliver the transgene into the cells.

Although there has been significant progress in the improvement of non-viral gene delivery systems, they are still much less efficient than viral vectors, especially for *in vivo* delivery [61].

1.5.1. Gene Therapy Clinical Trials

The first gene therapy clinical trial, for the disease adenosine deaminase (ADA) deficiency, was approved in 1990. This ex vivo therapy involved the *in vitro* genetic modification of the patient's own cells with the gene coding for ADA prior to re-administration of the cells into the patients' bloodstream (autologous cell transplantation). However, the current cost for genetically modifying autologous cells is very high and is a major hurdle for the wide-spread use of gene therapy [62, 63]. An *in vivo* gene therapy clinical trial in 1999 caused the death of Jesse Gelsinger by the injection of a novel gene construct into his liver. His death was believed to have been triggered by a massive immune response to the adenovirus carrier [64]. Many clinical trials were immediately put on hold and others were cancelled [2]. Another clinical trial published in 2002, reported the successful treatment of nine young boys with severe combined

immunodeficiency (SCID) using retro-virally transduced autologous stem cells. However, within three years, three of the boys developed T-cell acute lymphocyte leukemia [65]. Thus, one of the biggest problems for gene therapy using a viral based delivery system is the recognition of viral components by the immune system [66]. Until 2008, there have been 1472 gene therapy clinical trials that have been approved world-wide [67].

1.6. Gene Therapy for Hemophilia

Scientists have focused their efforts for testing gene therapy strategies on genetic diseases that are caused by single-gene defects such as cystic fibrosis, muscular dystrophy and sickle cell anemia. Hemophilia A also represents a combination of features that will most likely respond most favourably to gene-replacement therapy for numerous reasons. The clinical manifestations of the condition are completely attributable to the deficiency of a single gene product, FVIII, present in very low concentrations (~100μg/ litre of plasma). Slight increases to 2-3% of plasma FVIII levels should result in substantial reduction in symptoms, by specifically converting a severe hemophiliac into a phenotypically normal patient. Secondly, the expression of the FVIII gene is not tightly regulated within the body, making therapeutic levels in the circulation easier to achieve. Not only are many different cell types capable of producing coagulation factors like FVIII, the site of the protein synthesis is not critical to its function allowing further flexibility when choosing cell types and designing vectors for delivery. Last but not least,

there are very good animal models, including canine and murine, available for testing different gene therapy protocols [12] .

Significant progress has been made on the development of gene therapy for hemophilia A. There have been many recent advances in both *ex vivo* and *in vivo* gene transfer strategies that have reported physiological levels of human FVIII in normal animals as well as their hemophilic counterparts, including mice and dogs.

1.6.1. Gene therapy trials for hemophilia

There have been five different Phase I clinical trials utilizing different gene delivery systems including: a retroviral vector, an adenoviral vector, adeno-associated vectors, and a non-viral gene delivery method.

1.6.2. Non viral methods

One non-viral gene delivery method was also tested in a clinical trial [60]. This approach involved the transplantation of autologous fibroblasts into the patient after they had been modified *ex vivo* with a plasmid encoding the human FVIII cDNA. Animal studies using this method showed promising results, and the treatment was well tolerated during its Phase I clinical trial, however the trial only showed modest and temporary positive effects [6]. The loss of FVIII expression over time using non-viral methods can be attributed to several obstacles including, senescence of the implanted cells, promoter inactivation (due to possible viral origin), fibrosis around the transplanted cells and finally an immune response to the genetically-modified cells [6].

1.6.3. Viral vectors for hemophilia

Since retroviruses can only transduce dividing cells, liver-directed treatments using retrovirus for hemophilia are generally designed to induce hepatocyte cell division via partial hepatectomy. VandenDriessche et al (1999) were able to fully correct FVIII deficiency in a murine model of hemophilia A by infusing retroviral vectors into neonates [68]. Other preclinical studies for hemophilia A led to the beginning of a Phase I clinical trial for testing of a Moloney murine leukaemia virus (MMLV) retroviral vector encoding the BBD-FVIII [6]. Using retroviruses for FVIII delivery is efficient, as its genome can be integrated into the host genomic DNA, thereby providing the potential for long-term persistent gene expression. However, because of this integrating property of the virus, there is also a risk for insertional mutagenesis or insertional activation for adjacent genes. There is also a limitation as to which part of the body to target during treatment, given that retroviruses can only transduce dividing cells.

Gene therapy for hemophilia A has also seen frequent use of adenoviral vectors. These double-stranded DNA vectors demonstrate high transgene expression levels and transduction efficiency. However, due to the immunogenic properties of the adenoviral vectors, there often is only transient expression of the protein. Moreover, the effect of the immune response on transgene expression is increased in large animal studies (canine, primates) verses in murine models of hemophilia [69, 70]. To reduce the immunogenicity of these vectors, viral coding sequences can be eliminated during production.

Adeno-associated virus is a small replication defective, single-stranded DNA virus. Although there are no known symptoms or diseases associated with AAV infection (considered a benign virus), the small genome only allows for a small coding sequence capacity, making the construction of FVIII vectors more difficult than those for the smaller FIX gene. AAV vectors are also limited to post-mitotic (non-dividing) cells as it does not undergo vector genome integration. Much of the hemophilia gene therapy using AAV vectors has been directed at hemophilia B for FIX delivery.

The development of different lentiviral vectors has added much improvement to the area of retroviral therapy for hemophilia, with many advantages over first-generation vectors. These vectors, unlike most retroviruses, can transduce non-dividing cells making them an attractive for the transduction of hepatocytes and hematopoietic stem cells (HSCs). Chang *et al* (2006) recently showed the potential for lentiviral transduction of HSCs to produce FIX for the treatment of Hemophilia B.

Despite high transduction efficiency for most of these viruses, much of the *in vivo* therapy involving viral vectors typically result in transient expression of the transgene due to antigenic responses. Thus, the potential antibody response towards viral vectors remains a concern for human gene therapy trials.

1.7. Microcapsules

One way to avoid the cell-mediated immune responses directed against gene therapy treatment is to enclose the genetically modified cells in an implantable device, with pore size that prevents cell-mediated destruction of the modified cells. The pore size can be limited to <0.1μm as to prevent the diffusion of cytotoxic T lymphocytes, but large enough to allow for the delivery of a large molecule, like human FVIII, from the chamber [71, 72]. The immunoisolation technology was developed in the early 70s for implanting allogeneic and xenogeneic tissues. The technology for cell encapsulation represents a method by which cells secreting therapeutic products can be immobilized and protected within polymeric and biocompatible devices [71, 73]. With the advent of new and highly compatible biomaterials, these capsules can be continuously improved for drug delivery and gene therapy strategies. Cell encapsulation has the potential to eliminate the administration of immunosuppressant's and any serious side effects associated with these drugs [73]. The immunoisolation of recombinant cells is an attractive approach for hemophilia gene therapy and has already seen success in pre-clinical experimental treatments for diabetes [74], hyperparathyroidism [75], liver failure [76] and neurodegenerative diseases [77]. The implantation of allogeneic cells into a large number of patients can reduce the large cost of autologous implantation that other gene therapy strategies may demand. Furthermore, there is no modification of the host genome reducing the risk of mutation. The immunogenicity and biocompatibility of the materials chosen to create these microcapsules also needs be taken into consideration.

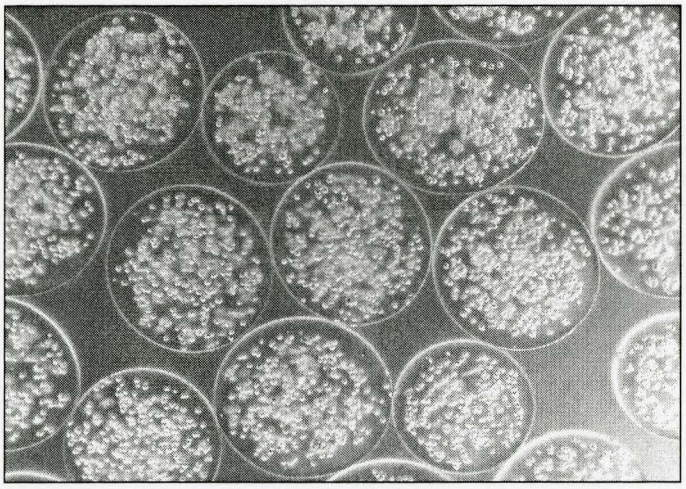


Figure 5 Alginate-poly-L-lysine-alginate (APA) microcapsules enclosing recombinant mouse myoblasts. Encapsulated cells are enclosed in microcapsules ~300-400μm in diameter. Capsules allow diffusion of FVIII from pores while preventing large immune cells from destroying the recombinant cells [78].

1.7.1. Alginate

Alginates are the most frequently used polymers for cell encapsulation due to their availability, biocompatibility, and easy gelling properties. Although other natural and synthetic polymers are being studied, none have performed as effectively as alginate [79, 80]. Alginates are natural polymers in seaweeds and bacterium, differing in composition depending on the source. The structure of alginate is an unbranched, linear co-polymer of β–D-mannuronic acid (M) and its C-5 epimer, α-L-guluronic acid (G) (Figure 6). The ratios between these residues have significant impact on the physical properties of alginate including biocompatibility, stability, mechanical resistance, permeability, biodegradability and swelling behaviour. Moreover, it has been shown that alginate

composition can also affect the immune response. Alginates with a high content in 'M' residues evoke an inflammatory response by stimulating monocytes to produce cytokines such as IL-1, IL-6 and TNF [81, 82]. Antibodies to alginates were found when high-M alginates were transplanted but not in the case of high-G alginates [83]. Still, the biocompatibility of alginate remains one of its most important of the gel properties, ensuring protection of encapsulated cells from cell-mediated immune responses of the host [73].

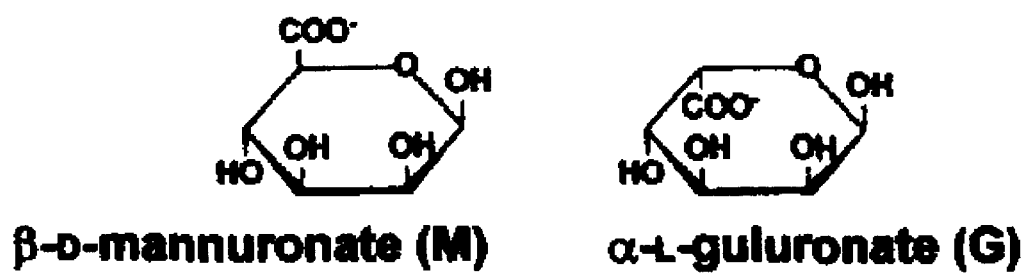


Figure 6 Alginate monomers. Alginates are a family of copolymers of 1→4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) of widely varying compositions and sequential structures [84].

1.7.2. Alginate-poly L-lysine-Alginate (APA) microcapsules

When alginate droplets are extruded into a solution containing divalent cations such as calcium or barium ions, the alginate is cross-linked to produce alginate beads. These negatively charged alginate beads are generally coated with a polycation to allow for stabilizing and controlling the molecular weight cut-off of the microcapsule membrane. The most commonly studied polycation for alginate coating is poly L-lysine

(PLL). Since the charged surface of PLL is known to be immunogenic and toxic [85], the alginate-PLL beads are further coated in alginate solution to form the complete alginate-PLL-alginate (APA) beads. This attempt to mask the immunogenic PLL does not, however, make the APA capsules fully biocompatible since they have been shown to activate complement system, and beads which do not include a PLL layer elicit a less severe response to the capsule than the complete APA capsule [86, 87]. The clinical successes of these APA capsules, in areas like gene therapy, will continue to improve as more research is conducted on improving its biocompatibility. In its current stage though, cell encapsulation shows great potential as a gene therapy treatment for hemophilia with several studies showing promising results for FVIII and FIX delivery.

1.7.3. Microcapsules for the treatment of Hemophilia

Microcapsules have successfully been used as vehicles for implanting recombinant cells that secrete FVIII for the treatment of hemophilia A [7] . They represent a feasible alternative to the standard treatment of intravenous infusions of plasma-derived or recombinant factors. Immunoisolation in this fashion can maintain viable cells secreting therapeutic levels of factor within the body for extended periods of time, eliminating the need for prophylactic treatment of FVIII. Myoblasts represent an attractive cell line to use for encapsulation, since they will not continuously proliferate they way other cell lines, such as fibroblasts do, but instead will terminally differentiate into myotubes [88, 89]. Encapsulated mouse myoblasts (C2C12 cells), have been engineered to secrete both clotting factors FVIII [7] and FIX [78, 90], and many other

proteins including adenosine deaminase (ADA), growth hormone, insulin, erythropoietin, and VEGF [73, 91-93].

Garcia *et al* (2002) engineered C2C12 myoblasts, a transformed murine cell line, to secrete the BDD version of human FVIII in C57BL/6 normal mice. Although therapeutic levels of FVIII were observed, (up to 22ng/ml), expression was transient and began to decrease after day 1. The transient delivery was accompanied by the development of an antibody response against FVIII starting on day 14. However, in addition to antibodies, other factors were responsible for the decline in FVIII. Encapsulated C2C12 myoblasts were also implanted in immunodeficient SCID mice to determine other possibilities. It was found that the FVIII delivery in these SCID mice was also transient suggesting that clearance of the protein was not solely caused by antibodies. Encapsulated C2C12 cells were implanted into C57BL/6 mice, and FVIII mRNA levels were measured to determine whether the observed decline in antigen level was due to decreased mRNA levels from vector inactivation *in vivo*. A 5-fold reduction in FVIII mRNA levels, compared to pre-implantation levels, was found after retrieval of encapsulated C2C12 cells. Therefore, the decline in plasma FVIII levels was primarily due to a decrease in FVIII mRNA caused by transcription repression. Antibody induction, however, is a challenge that still needs to be tackled.

Thus, the work carried out by Garcia *et al* (2002), demonstrates the potential feasibility of using microcapsules for the delivery of FVIII in the treatment for hemophilia A. However, many obstacles need to be overcome for the optimization of this

strategy. The transient delivery of FVIII due to viral vector inactivation suggests the need for improved expression vectors. Moreover, C2C12 is a transformed cell line that has been shown to develop tumours in nude mice [94]. Therefore, a safer alternative cell line must be tested, especially for microcapsule treatment of hemophilia A in humans.

1.7.4. Mouse primary myoblasts (G8) & blood outgrowth endothelial cells (BOECs)

G8 myoblasts are derived from primary fetal mouse tissues and are a safer cell line of choice than C2C12 myoblasts. In contrast with findings using C2C12 cells, encapsulated G8 myoblasts engineered to secrete FIX in hemophilia B mice were able to safely deliver therapeutic levels of FIX with undetectable levels of antibodies. More importantly, the delivery of FIX from engineered G8 myoblasts was sustained for at least 120 days. Furthermore, they did not induce the generation of myoblastic tumours in nude mice [8].

There are other potential somatic cells for hemophilia gene therapy. Although there has been interest in using blood outgrowth endothelial cells (BOECs) in vascular regeneration, these cells may also be an alternative target for expressing FVIII. In a previous preclinical study, BOECs were modified to express human FVIII and introduced into NOD/SCID mice. This resulted in expression of therapeutic levels of human FVIII in the plasmas of the treated mice [95]. BOECs deliver sustained therapeutic levels of FVIII from a scaffold, in both NOD/SCID and hemophilia A mice [96].

Thus, these two cell lines have the potential for delivering high levels of FVIII from APA capsules. It is also important to study how their regular cell behaviour – viability, internal cell composition, and proliferative capacity – are modified by encapsulation.

1.7.5. Cell viability, granularity and proliferation

One of the main goals of a cell encapsulation strategy for hemophilia A, is to optimize the ability of the cells to secrete FVIII. An important factor that has a direct influence on secretion is cellular viability. Cell viability refers to the *extent* to which the modified cells remain alive and healthy. Thus, employing an accurate method for viability calculation is of utmost priority in gene therapy experiments to obtain a reasonable interpretation of cell secretion results.

Many assays for viability can only report the percentage of absolute living cells, but not the health of these living cells. Flow cytometry offers a measurement for both. Flow cytometry is an extremely sensitive technique that can be used to examine several thousand particles every second, and can actively separate and isolate particles with specific properties. Flow cytometry offers a two parameter analysis of cell viability; it can identify living cells (determined by the intensity of fluorescent chemical attached to the cell), and also the proportion of the living cells that are healthy (determined by the degree of side scattering of the cells) [97]. The degree of side scatter on a flow cytometry plot depends on the inner complexity of the particle (i.e. shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness). In essence, side scatter is a

measure of the relative granularity of the cell and thus can be used as an indication of its health. As a cell deteriorates in health, it begins to engulf material internally, forming vesicles. This results in a measured side scatter greater than that of a healthy cell. Therefore, an increase in granularity can indicate a decrease in the health of a particular sample of living cells (FACS MultiSET System, BD Biosciences).

Flow cytometry analysis has never been utilized for the measurement of viability of encapsulated cells. It offers a highly accurate and objective method of viability calculation. Moreover, it allows for an additional level of analysis for cellular health (or granularity), which other methods of viability cannot assess. Thus, it is worthwhile to investigate flow cytometry as a novel method for reporting viability of encapsulated cells.

Along with cell viability, cell proliferation is another indicator of normal cell behaviour. Proliferation of certain cell lines such as myoblasts, is limited within the microcapsule [88, 89]. However, the actual proliferation profiles of encapsulated cells *in vitro* or *in vivo* have never been examined. The introduction of the division tracking dye, carboxyfluorescein diacetate succinimidyl ester (CFSE), has made it possible to examine the number of cell divisions during proliferation [98]. Encapsulation may affect the proliferation behaviour of cells. Studying the changes in proliferation between free cells, encapsulated cells *in vitro* and encapsulated cells *in vivo* may provide additional insight into how to optimize encapsulated cell secretion.

1.8. Thesis Rationale

Results of studies using G8 myoblasts and BOECs demonstrate the strong suitability of these cell lines for FVIII delivery by hemophilia A gene therapy. The Hortelano lab has obtained G8 and BOECs cell lines transduced with the pLenti-TM-B domain deleted cFVIII vector plasmid from Dr. David Lillicrap's laboratory in Kingston, ON. The cFVIII in this plasmid, under the control of the endothelial cell-specific thrombomodulin promoter (EF1-a), has been previously shown to maintain high levels of cFVIII *in vivo* [96]. Unlike proliferative cell lines, myoblasts are more suitable for encapsulation since their differentiation limits their growth within the microcapsular space. This characteristic of myoblasts prevents crowding of the microcapsule and allows space for the diffusion of the large FVIII molecule through the pore and also promotes the long-term viability of the enclosed cells. G8 myoblasts of primary origin are a safer cell line than C2C12 cells; as the latter induces an undesirable immune response and tumour generation [7, 94]. BOECs have the potential for extended longevity, and generating of daughter endothelial cells. Thus, G8 myoblasts and BOECs may be suitable for encapsulation and the treatment of a chronic disease such as hemophilia.

The current work will focus on delivering implantable microcapsules enclosing engineered G8 myoblasts secreting FVII, into hemophilia A mice with a goal for sustaining therapeutic levels of FVIII. Another goal of this work is to examine the behaviour of BOECs within capsules *in vitro*, as they have been previously shown to deliver therapeutic levels of cFVIII *in vivo* via a matrigel scaffold [96]. The effect of

encapsulation on the ability of these cell lines to secrete cFVIII will be examined. Further, yet another goal is to develop a novel flow cytometric assay for measuring cell viability in order to accurately assess both the viability and granularity of encapsulated myoblasts and BOECs. Finally, the cell division tracking CFSE dye will be employed to investigate the proliferation profiles of free and encapsulated myoblasts *in vitro* and *in vivo*.

1.8.1. Specific Project Objectives

The thesis will specially focus on the ability of encapsulated G8 myoblasts to deliver functional FVIII after implantation into hemophilia A mice. Novel flow cytometric assays will be used to provide a thorough assessment of encapsulated cell viability and proliferation to determine to what extent these factors influence the cells' ability to secrete FVIII. The following objectives will be carried out in order to fully characterize the potential of APA capsules as a gene therapy delivery system.

Objective 1: Develop a reliable and accurate method for measuring the viability of encapsulated cells by flow cytometry.

Objective 2: Characterize encapsulated G8 myoblasts and BOECs *in vitro*

Objective 3: Study the delivery of cFVIII by encapsulated G8 myoblasts *in vivo,* using C57BL/6 and FVIII-deficient KO mouse models.

Objective 4: Investigate the proliferation profiles of encapsulated cells verses free cells *in vitro* and *in vivo.*

2. MATERIALS AND METHODS

2.1. Cell lines and culture

G8 cells are a myoblast cell line derived from the fetal skeletal muscle tissue of Swiss Webster mice (ATCC, CRL-1456). G8 cells and BOECs transduced with pLenti-TM-BDDcFVIII, were obtained from the David Lillicrap lab in Kingston, ON. Myoblasts were cultured on 0.01% collagen coated plates in Dulbecco's modified culture medium (DMEM) supplemented with 1.59 g/L sodium bicarbonate ($NaHCO_{3,}$), 20% fetal bovine serum (FBS), 1% streptomycin (100 µg/ml) and penicillin (100 µg/ml) (P/S). BOECs were cultured on 0.01% collagen coated plates in MCDB media containing complete endothelial cell growth kit (Invitrogen), and 10% FBS and 1% (P/S). Both cell lines were grown on 10 cm polystyrene tissue culture plates at 37°C with 5% CO_2. Cells were subcultured every 2-3 days no higher than a 1 in 4 dilution.

2.2. Encapsulation

Cells were grown on collagen coated plates until they were approximately 80% confluent. Once the media was aspirated, the cells were washed with PBS and subsequently harvested using tripLE (Gibco). After detaching the cells, culture media was added to stop trypsinization reaction. Cells from each plate were collected in 50 mL falcon centrifuge tube and spun at 1000 g for 10 min at 4°C (Mendel Scientific Co. Ltd). The supernatant was removed and the cells were re-suspended well in 0.9% NaCl. A sample of this mixture was diluted1:10 in isoflow solution to be used in cell count, and

the remaining suspension was removed by centrifugation as noted above. The supernatant was removed and cells were re-suspended, using a 16 G needle attached to a 20 mL syringe, this time in 1.7% low viscosity filtered alginate (Improved Kelmar, ISP Alginates, San Diego, CA, USA) to achieve a concentration of approximately 5 x 10^6 cells/ml. (*important note: avoid introducing air bubbles into the cell/alginate mixture as it may affect size of capsule). The syringe was attached via luer lock to the encapsulator tubing and placed into the syringe pump. Alginate/cell mixture was infused at a rate of 0.9 mL/min into rubber tubing and extruded as droplets through a 0.35 mm needle into a solution of 1.1 % $CaCl_2$ to form calcium-alginate beads 300-500 μm in size. Microencapsulation was performed with an encapsulator (Nicso Engineering Inc., Zurich, Switzerland) at 7kV. Once the syringe was empty, the beads were transferred onto a 0.45μm filter to undergo a series of washes consisting of $CaCl_2$, 0.1% CHES, 0.9% NaCl, 0.05% poly-L-lysine, 0.03% alginate, and serum-free culture media. The outer layer of alginate was cross-linked with 0.05% poly-L-lysine (PLL; Sigma, St. Louis, MO, USA) for 6 min and then coated with a second layer of 0.03% alginate. After the procedure, the resulting microcapsules were incubated in serum-free media under regular tissue culture conditions overnight before their being implanted into the mice. Non-implanted microcapsules were cultured *in vitro* conditions with full growth media that was changed every 2-3 days.

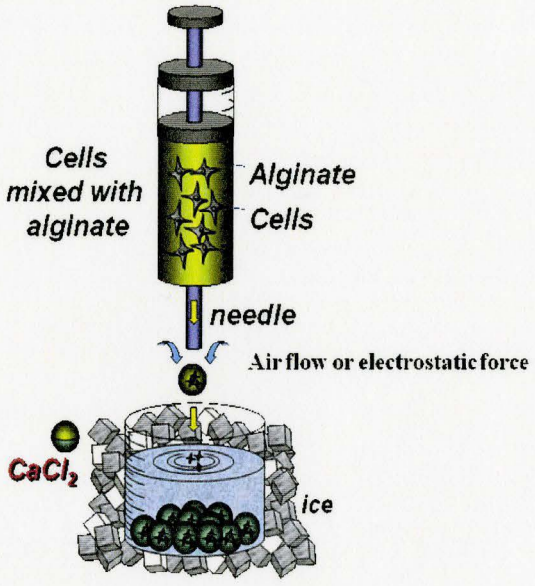


Figure 7 Cartoon representation of encapsulation setup. Cells mixed with alginate are pumped through a syringe via a pump (not shown). The solution travels through the rubber tubing to the encapsulator (not shown) where it passes through a small charged needle to form a very small stream of droplets that fall into a solution of $CaCl_2$ (Wen, JP)

2.3. Cell Viability

The viability of cells refers to the extent to which those cells are functioning normally as living cells. After the cells had undergone encapsulation, their viability was measured using two different techniques: trypan blue exclusion and flow cytometry. Since flow cytometry can yield single cell data, for both live and dead cells, it allowed for a more precise and consistent assessment of cell viability.

2.3.1. Trypan blue

Trypan blue is a diazo dye used to selectively colour dead cells or tissue blue. Cells that are alive have intact cell membranes that prevent the diffusion of the dye into them, remaining colourless. Since living cells selectively determine compounds which are allowed to pass through their membrane, a viable cell will not allow trypan blue to be absorbed. In contrast, dead cells allow the dye to transverse their membranes by diffusion, to thereby display a distinctive blue colour under the microscope. This staining method, also described as dye exclusion method, was used to determine the viability of the cells within capsules in the study.

A small sample of capsules (5-30μl) was placed on a glass slide and any excess media was aspirated off the slide so that a drop of trypan blue solution (Invitrogen, USA) could be added. A cover slip was placed directly over top of the capsules, and gently pressed to break the capsules and release the enclosed cells into the blue dye. Live (transparent) and dead (blue) cells were assessed and quantified by light microscopy.

2.3.2. Flow Cytometry

Flow cytometry is fluorescence-based method used to analyze a heterogeneous population of particles/cells. It is an extremely sensitive technique used for counting, examining and sorting microscopic particles that sorts several thousand particles every second in real-time. Although it is commonly used for antibody-based separation of cells with specific cellular markers, the availability of a flow cytometer and its ability for high

throughput single cell analysis made it a powerful tool with which we could more accurately measure cell viability than by trypan blue exclusion.

To our knowledge, this is the first study that has used flow cytometry to evaluate the viability of alginate-poly-L-lysine-alginate (APA) encapsulated cells. We proposed and developed a novel flow cytometric assay to examine the viability, granularity, and proliferation of cells within the capsules.

2.4. De-encapsulation of APA capsules for flow cytometry

Before the enclosed cells could be assessed for their viability, the capsular material had to be removed to prevent large debris from clogging the flow cytometer. Instead of crushing the capsules to release the cells, a modified version of a recently proposed method for de-encapsulation was used to release the cells and eliminate cell death that may have resulted from crushing the capsules between glass slides [99]. In order to do this, a protocol from de Groot *et al* (2002), was optimized for the complete removal of the alginate-poly-L-lysine layer around the cells. This approach utilizes trypsin which cleaves poly-L-lysine peptides at the carboxyl end of each lysine residue, and EDTA, a chealting agent which depolymerizes the alginate by chealating the Ca^{2+} ions in the polymer. Trypsin also dissociates confluent cells, if clumped, into single cell suspensions.

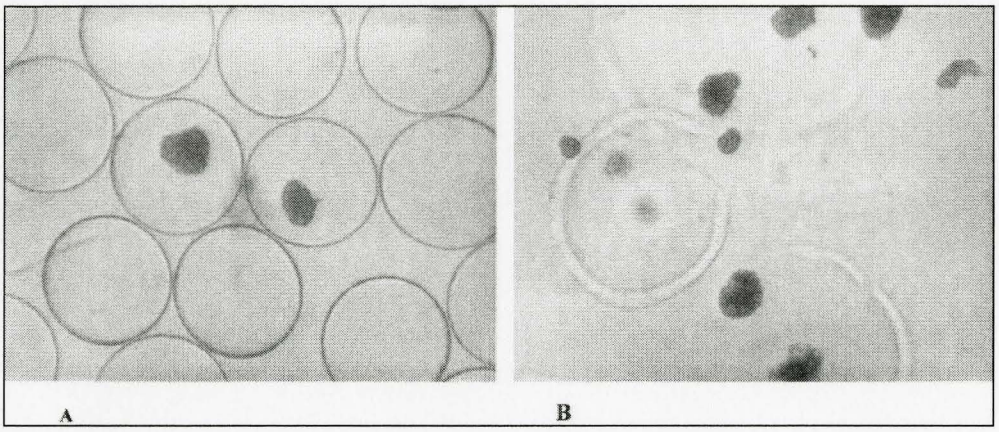


Figure 8 Trypin/EDTA removal of PLL and alginate capsular layers. (A) Encapsulated islets. (B) De-encapsulated islets plus remaining broken capsules [99]

Cells were labeled with a fluorescent dye called calcein AM (Invitrogen). Calcein transports across the membrane into a cell where it can be cleaved by intracellular esterases. Cleavage results in the molecule getting trapped inside and the production of an intense green fluorescence that is emitted by the cell. Only live cells this ubiquitous intracellular esterase activity, and are thus able to convert calcein into its fluorescent form. As dead cells lack active esterases, only live cells are labeled (Molecular Probes, Invitrogen).

Briefly, 150 µl of capsules were added to 5 ml of trypsin/EDTA (Gibco) and allowed to incubate in a 37°C water bath, gently shaking for 30 min. Any unbroken capsules or cell clumps were sheared by pipetting a few times. Once the cells were successfully de-encapsulated, with no detectable capsules, the solution was centrifuged at1000 rpm for 3min to isolate the cells in a pellet. The supernatant was aspirated until

approximately 100 µl of solution remained, to which 400 µl of 0.5% BSA in PBS (Ca^{2+} and Mg^{2+} free) was added and mixed with the cells. Subsequent staining was performed with 20 µl 7- aminoactinomycin D (7-AAD) and 1 µl 50 nM calcein, at room temperature in a dark environment, and the solution was allowed to incubate with the dyes for 20 min before being passed through a 40 µm filter into a flow tube. To confirm the effectiveness and safety of this de-encapsulation protocol, the effect of trypsin/EDTA on the viability of free cells (non-encapsulated cells) at 37°C for 30 min was also tested to ensure that the viability were the same as control cells – i.e. free cells incubated in regular media at the same conditions.

2.5. Cell proliferation assay

In order to examine cell proliferation patterns of both free cells and those within the capsules, cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) – a fluorescent cell staining dye. CFSE is a simple and sensitive technique for analysing multiple parameters of cells. This dye, which is retained within cells, allows for the examination of specific populations of proliferating cells and can survive for 7-10 successive cell generations. Once the CFSE molecule diffuses inside the cells, its acetate groups are cleaved by intracellular esterases converting it into a fluorescent membrane-impermeable dye. Since CFSE is retained by the cell in the cytoplasm, each round of cell division decreases the relative fluorescence intensity of the dye by half [98].

The cells to be labeled were re-suspended in PBS to a concentration of 1-5x10^6 cells/mL. Staining was performed at 5 µl of 0.5 mM CFSE per ml of PBS. The cell solution was mixed rapidly to ensure an even distribution of dye and staining was allowed to take place for 10 min at room temperature. An excess of wash buffer (5% FBS in PBS) or media was added to stop the reaction. The newly labeled cells were re-suspended in media or mixed with alginate to carry on an encapsulation experiment. A novel flow cytometry analysis, like the one developed for viability measurement, was also performed for assessing proliferation.

2.6. Animal Experimentation

In order to evaluate the efficacy of cell encapsulation as a treatment for hemophilia A, *in vivo* experiments were carried out in both hemophilia A and normal mice. McMaster University maintains a colony of hemophilia A C57BL/6 mice generated at the Salk Institute , La Jolla, CA, USA. Non-hemophilic, C57BL/6 mice were purchased from Charles, River (Montreal, Canada). For *in vivo* implantation of capsules as well as extraction of blood samples, mice were anaesthetized with isofluorane (Bimeda-MTC, Animal Health Inc., Richmond Hill, Canada) in a small anesthetic machine (Med-Vet Anesthetic System Inc., Toronto, Canada). All of the animal procedures conducted during experimentation were performed in accordance with McMaster University's Animal Ethics Guidelines.

2.6.1. Microcapsule Implantation and Retrieval

Once made, the microcapsules were incubated in regular tissue culture conditions overnight before being implanted into the mice. The capsules were washed several times with PBS to ensure complete removal of culture media whose serum may trigger an immune response in the mice. After being thoroughly washed, excess PBS was removed and the capsules were loaded into a 20ml syringe using a 16G needle. Microcapsules were injected intraperitoneally (IP) using a G18 catheter. Mice in all experiments were injected with 20 x 10^6 cells each (4mL of microcapsules). The microcapsules were kept inside the mice for 4 to 6 weeks.

Upon retrieval of the capsules at the end of the experiment, mice were sacrificed by cervical dislocation while anaesthetized. The capsules were re-suspended in PBS and washed several times to remove free floating macrophages and lymphocytes in the peritoneum, and other debris that were often retrieved along with the capsules. After retrieval, the capsules were cultured *in vitro* under standard tissue culture conditions to monitor FVIII secretion from media aliquots at specific time points.

2.6.2. Immunoassays

Blood samples (70 μl) were taken at regular weekly intervals by retro-orbital bleeding (from the orbital plexus) using capillary tubes. Sodium citrate was used as the anticoagulant at a ratio of 1:9 for the volume of blood collected. Plasma was obtained for each mouse by centrifugation of the blood sample at 13,000g at 4°C for 10min, and stored

at -70°C. FVIII protein levels were assayed with an ELISA and by aPTT for FVIII:C activity.

2.7. Estimating FVIII concentration by enzyme-linked immunosorbant assay (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is highly specific for the target protein, sensitive, and convenient to use. An antibody against FVIII is immobilized on an inert solid (polystyrene). The sample to be assayed (either mouse plasma or culture media) is added to the antibody-coated surface and any other proteins are subsequently removed through a washing step. The FVIII bound to the antibody is reacted with a second detecting antibody to which an enzyme is attached. Once a substrate is added, the amount of fluorescence it produces via enzymatic activity is proportional to the amount of protein present.

2.7.1. FVIII:Ag ELISA

A FVIII ELISA kit (Affinity Biologicals, Ancaster, Canada) was used to detect FVIII from *in vitro* and *in vivo* experiments by assaying cell culture and mouse plasma, respectively. Normal pooled human plasma (George King Biomedical, Overland Park KS, USA) and normal pooled canine plasma (Central Animal Facilities, McMaster University) were used to derive the standard curves for the detection of human FVIII and canine FVIII respectively, delivered into mouse plasmas. Normal human plasma contained between 100-200 ng/ml FVIII. Normal canine plasma was approximated to

contain 1IU/ml of canine FVIII. The gravimetric concentration of canine FVIII consisting of 1unit/mL of plasma is unknown.

2.7.2. FVIII:Ag ELISA setup for *in vivo* samples

Sheep anti-cFVIII antibody (Affinity Biologicals) was diluted 1:100 in coating buffer (50 mM sodium carbonate, pH 9.6) and 100 µl was added to each well. After an overnight incubation at 4°C, the unbound antibody was discarded by suction and the plate was washed 3X with PBS-tween (0.1% v/v, pH 7.4). The samples along with the reference standards were added to each well and allowed to incubate for 2 hours at room temperature (RT).

Standards were made by serial dilutions of canine plasma in dilution buffer. The highest standard (deemed to be 100% FVIII) was prepared so that the canine plasma had a final dilution of 1:5. Hemophilic or normal mouse plasma (20 µl) was also added to each standard to reproduce the conditions of the test samples, ensuring that any difference in FVIII measurement between the standards and the samples was only due to the presence of the canine FVIII delivered in the treatments. The remaining volume was brought to 100 µl by dilution buffer (e.g 100% = 20µl canine + 60 µl dilution buffer + 20 µl hemophilic plasma; canine: total = 1:5). Two-fold serial dilutions were carried down to 0.097% (0.002 ng or 0.976 mU). The samples from mice to be tested were also diluted 1:5 in dilution buffer (20 µl sample + 80 µl dilution buffer).

Following the incubation of the samples, the plate was once again washed, and 100µl of pre-diluted detecting antibody was added to each well and allowed to incubate at RT for 1.5 hours. A third wash step was performed after the removal of the detecting antibody (that had not bound the FVIII already immobilized to the wells by the coating antibody), and 100µl of the substrate o-phenylenediamine (OPD, 5mg tablet) diluted in substrate buffer (Citrate-Phosphate buffer, pH 5.0) with 30% hydrogen peroxide was immediately added to each well. The colour reaction was allowed to develop for 10-30 min at RT before being stopped with 2.5M H_2SO_4. The absorbance at a wavelength of 490 nm was then measured.

2.7.3. FVIII:Ag ELISA setup for *in vitro* samples

Standards for testing *in vitro* samples contained serial dilutions of normal plasma (human or canine) in the culture media from which the sample was taken from, instead of dilution buffer. Two-fold serial dilutions were carried down to 0.097% (0.002ng or 0.976mU). Media samples for *in vitro* assays were added undiluted (100µl). The protocol for the ELISA was the same as the test for *in vivo* samples.

2.8. Activated partial thromboplastin time (aPTT)

In order to determine whether the FVIII delivered to the mice was coagulantly active, the plasma was tested in a functional assay known as the activated partial thromboplastin time (aPTT). This test measures the time required for a sample of plasma to clot and will be prolonged if the blood lacks anyone of the contact clotting factors. The

intrinsic pathway requires phospholipids, an activator (such as silica), calcium and clotting factors to become activated. Calcium and phospholipids are required for the tenase and prothrombinase complexes to assemble on the phospohlipids and to function. Calcium also mediates the binding of the complexes (through the terminal carboxy glutamic residues on FX/FXa and FIX/FIXa) to the phospholipid surfaces expressed by platelets. When performing the assay, the aPTT reagent provides both the activator of contact factor and coagulant phospholipids while plasma deficient in FVIII provides the remaining clotting factors. Once the test sample is added, calcium chloride is required to initiate FIX activation by FXIa to initiate and propagate the clotting reaction. The time for a clot to form is measured and is indicative of the concentration of FVIII in the test plasma of interest.

Plasma samples drawn from the mice were assayed for the presence of functional FVIII expression by examining clotting time using a coagulation analyser (ST art4, Diagnostica Stago). Normal human or canine plasma (McMaster University pool) was used to generate a standard curve to allow quantification of FVIII activity in the mouse samples. Plasma samples (from blood collected in citrate) and FVIII deficient plasma were thawed at 37°C for 15 min and placed on ice. The plasma sample was mixed with veronal buffer and FVIII deficient plasma in a ratio of 1:9:10, respectively, and incubated for 15 min on ice. A volume of 100 µl of this solution was added to 50 µl of aPTT reagent (containing activator and phospholipids) in an assay cuvette with a stainless steel ball. After 180s of incubation at 37°C, the aPTT reaction was initiated by the addition of

50 μl of 20 mM $CaCl_2$, and the time until clot formation was measured as the time when movement of stainless steel ball ceased. The clotting time observed was proportional to the FVIII:c concentration in the test samples.

2.9.Tail-clip assay: hematocrit measurement

Treated and control hemophilic mice were subjected to a tail-clip on day 7 after capsule implantation, as a way to evaluate protection against blood loss after severe trauma. Blood loss was assessed by measuring the hematocrit following the tail clip.

Tail-transection was performed using a sterile stainless steel surgical blade (Almedic, Montreal Canada) and the mice were subsequently returned to their cages. Three days later (or 10 days after implantation), a blood sample was collected from both treated and control mice via heparinized capillary tubes. The samples were transferred to micro-hematocrit tubes and plugged at one end using critocap. Samples were centrifuged in a hematocrit centrifuge (Oxford Labware, St Louis, MO, USA) at 11809rcf for 5 min.

The tubes were placed on the Micro-Hematocrit Capillary Reader Chart with the bottom edge of the critocap just touching the edge below the zero percent line. The bottom of the column of blood was then placed so that it was at zero percent line. The tube was slid along the chart until the meniscus of the plasma intersected the 100 percent line. The height of the packed red cell column was then read directly as percent red cell volume.

2.10. Statistical Analysis

A statistical significance of the difference between means was determined using the 2-tailed Student's *t* test. Experiments were conducted at least in triplicate. The error graphs display the standard deviation of the data.

3. RESULTS AND DISCUSSION

3.1. Development of a novel flow cytometry based assay to examine encapsulated cell viability

The quantification of cell viability is essential in cell-based studies for reporting cell growth or cell death. Reporting cell viability as accurately as possible is necessary in gene therapy cell studies, as many strategies employ genetically modified cells for secreting the protein of interest. The strategy proposed in the current work relies on the ability of encapsulated cells to secrete therapeutic levels of cFVIII. The viability status of these cells will help to elucidate changes in secretion. Thus, as one of the direct indicators for cell secretion, it was critical that a reliable and accurate method was available by which we could determine cell viability.

Many viability assays are based on one of two characteristic parameters: metabolic activity or cell membrane integrity of healthy cells [100]. The dye-exclusion method of assessing viability takes advantage of the ability of healthy cells with uncompromised cytoplasmic membrane integrity to exclude dyes such as trypan blue. However, the drawbacks associated with this type of viability assay led to the development of a superior flow cytometry method to measure encapsulated cell viability.

3.1.1. Trypan Blue

Trypan blue exclusion has been previously used to assess the viability of the FVIII secreting encapsulated cells [7-9, 78, 90, 94] Although trypan blue exclusion has been employed as a common test for cell viability, there are many reasons why this method can be highly subjective. The count of live and dead cells in a field of view can vary significantly depending on what is perceived by the viewer to be a healthy cell [101]. Since there are no size restrictions, it is often difficult to distinguish smaller, viable immune cells from regular cells, both which appear bright and transparent. Also, the adherence of inflammatory cells (post-retrieval from mice) to the outside of membranes creates a large mass of cells, making it difficult to obtain an accurate count. Finally, trypan blue relies on the accuracy of the individual counting the cells, and is therefore subject to human error and inconsistency [101] This method of measuring cell viability is highly subjective toward the examiner It was thought to be crucial in our experiments to have a viability test that more accurately reports the true behaviour of the cells within the capsules, as viability data can affect the interpretation of secretion results.

3.1.2. Flow Cytometry: A novel approach for measuring cell viability

We developed a novel protocol for measuring cell viability within capsules using flow cytometry Flow cytometry is an extremely sensitive technique used for counting, examining and sorting microscopic particles and can analyze several thousand particles every second in real-time, in comparison to trypan blue where the count is dependent on the individual and his/her field of view Although the premise of trypan blue exclusion is

valid, the large number of cells and the sensitively detected by the flow cytometer allows for a more quantitative and objective method of viability assessment. To our knowledge, no group has studied the viability of APA encapsulated cells using this technique. Thus, we proposed a unique flow-based method of viability analysis for the thorough examination of cell viability, as we believe it to be superior to that of dye exclusion methods.

Before cell viability could be assessed by flow cytometry, enclosed cells had to be released from the microcapsules. Standard procedure for trypan blue exclusion involved crushing the capsules between a glass slide and cover slip and subsequently staining the released cells with the blue dye. It was suspected that a subset of cells might be killed during this crushing process, contributing to a decrease in cell viability. Moreover, our flow-based analysis required that the cells be suspended and labeled in solution before being analyzed through the flow cytometer – also not possible using the crushing method. Thus, in order to minimize cell death by cover slip compression, and to maintain cells in solution, a de-encapsulation protocol from de Groot M *et al* (2002) was adapted. Our protocol utilized trypsin and EDTA to cleave the PLL and depolymerize the alginate, respectively, and also to dissociate cell aggregates. This procedure allowed us to discard the dissolved capsular material, and stain the cells (directly in solution) with the fluorescent label to assess viability. The fluorescent label used in our experiments was the acetomethoxy derivative of calcein; calcein AM (see materials and method section 2.4)

3.1.3. Method of analysis

Flow analysis was carried out using the Cytomics Flow Cytometer 500 (Beckman-Coulter). The viability of free (non-encapsulated) cells for each cell line (G8 or BOEC) was first measured, in order to establish an absolute value to which the viability of encapsulated cells could be compared. The calculation for free cell viability was based on the intensity of calcein-labeled cells. Only those cells that were brightly labeled with calcein (as determined by intensity scale) were considered alive, and this percentage of cells was deemed the absolute viability of free cells for that cell line ($Total_{free\ cells}$). Cells which had very low to dim labeling of calcein were considered dead or recently dead with residual enzyme activity (Figure 9, panel I).

Next, a specific subsection of the entire FF/SS plot (Figure 9, panel II) was established as the healthy live cell box (box A), where only those cells >20 μm would be considered healthy and included in this box. A similar box (box B) was established for the unhealthy or dead cells on the plot, comprised of all cells between 5-20 μm (Figure 9, II). The creation of these size restrictions was to ensure that immune cells, majority of which are < 20 μm would not appear in the healthy live box or in the count for viability

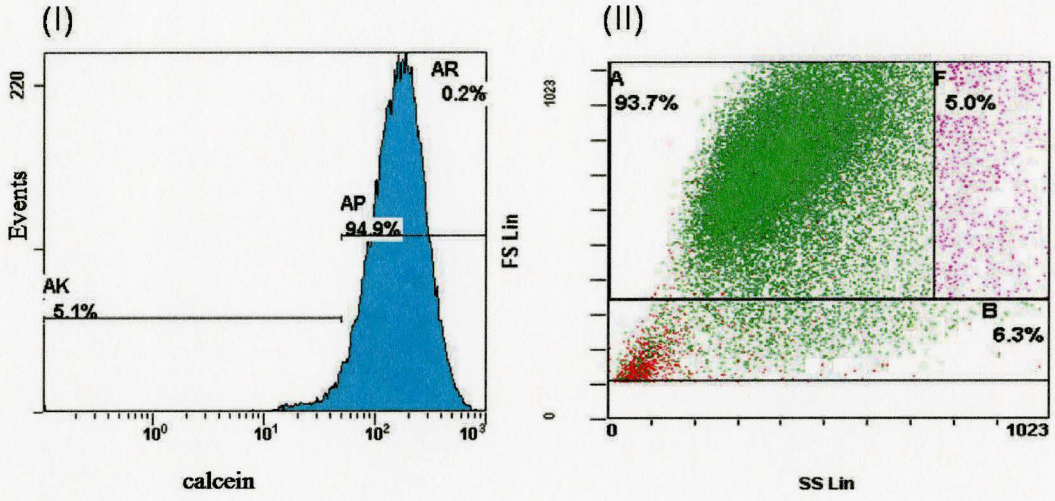


Figure 9. Flow cytometry of free (non-encapsulated) G8-cFVIII cells. (I) Intensity plot of all cells labelled with calcein. AP bracket represents fraction of cells which are brightly labelled (green in panel II), while AK bracket represents the fraction of cells which are dimly labelled (red in panel II). (II) The corresponding FS/SS plot of all calcein labelled cells from panel I. Box A represents the healthiest fraction of all cells and box B represents the unhealthy fraction of all cells, based on size. Box F (subset of Box A) represents the population of granular cells.

Panel I in Figure 9, is a representative calcein intensity plot for all G8-free cells labelled with calcein. 94.9% (AP bracket) of all cells are brightly labelled (appear green in panel II) and deemed to be viable. This was taken to be the $Total_{free\ cells}$ value for G8 cells. 5 1% (AK bracket) represents the fraction of cells that are dimly labelled with calcein and are not included in the $Total_{free\ cells}$ value (appear red in panel II). The A box (panel II of Figure 9) represents the healthiest fraction of all cells based on size (>20 μm),

with 93.7% of calcein labelled cells appearing in this box. The B box, (panel II of Figure 9) represents majority of the unhealthy or dead fraction of cells based on size (5-20 μm).

These size cut-offs for the lower and upper limits were estimated by testing various fluorescent beads of known size (CountBright™, Invitrogen), as well as by the location of healthy cells (Figure 10)

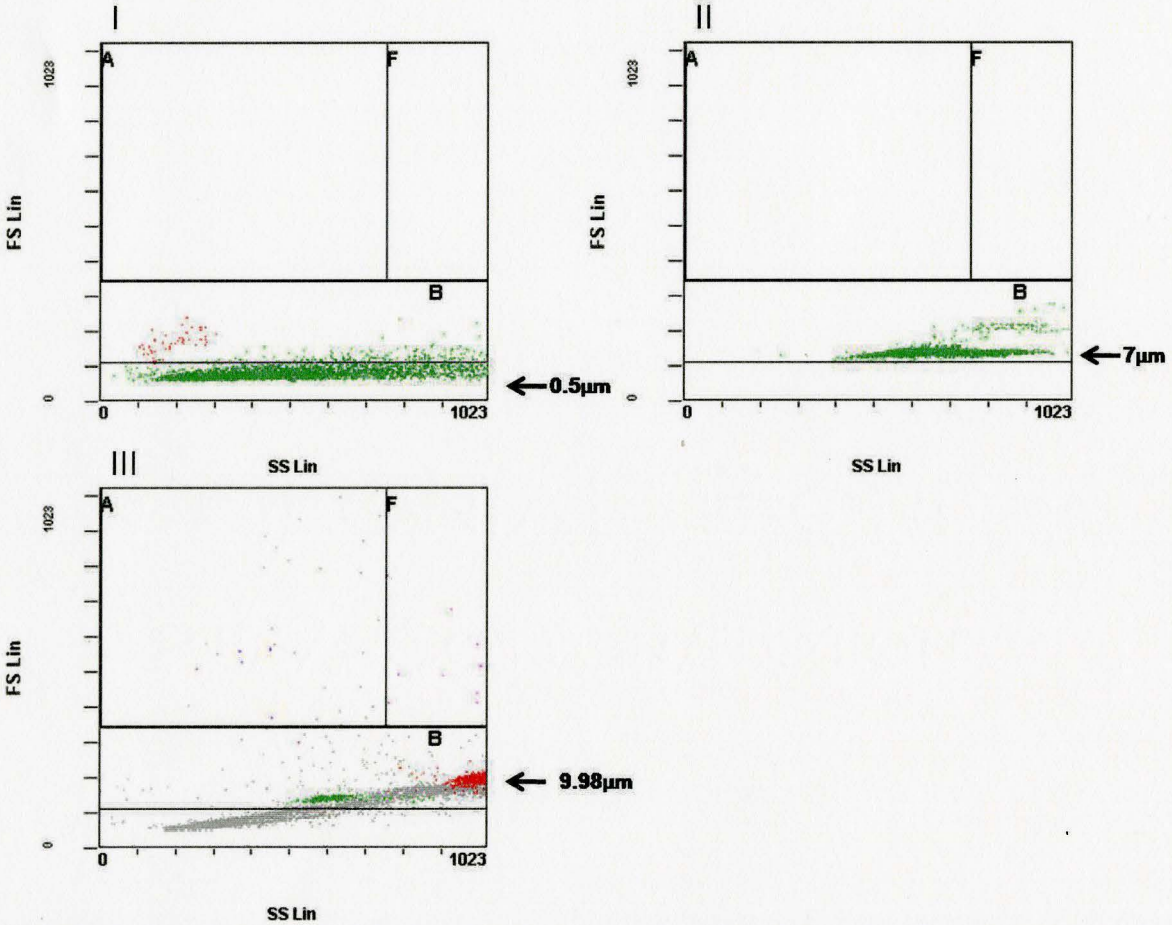


Figure 10 FS/SS plot of CountBright beads of various sizes for determination of upper and lower size limits. (I) bead size = 0.5μm (II) bead size = 7μm (III) bead size = 9.98μm

As previously described, these size restrictions eliminated small immune cells, or very small cell fragments or debris to be considered in the count for viability. Therefore, only the brightest (expressing calcein at high intensity) and healthiest population (>20 μm) of cells, as deemed by Box A, were considered to be alive.

3.1.4. Viability calculation

The calculation for viability for the sample of interest was found by the difference between the percentage of live cells in Box $A_{free\ cells}$ and percentage of live cells in Box A_{sample}, subtracted from the absolute viability of free cells (equation 1).

$$\text{Percent Viability} = (\text{Total}_{free\ cells} - (\text{Box } A_{free\ cells} - \text{Box } A_{sample}))$$

Equation 1 Viability calculation for encapsulated cells labeled with calcein. $\text{Total}_{free\ cells}$ and Box $A_{free\ cells}$ remain constant for a specific cell line. Only the Box A_{sample} value changes for each test sample.

This method of viability calculation is termed "calcein loss" as it equates the relative loss in calcein intensity from the control (free cells) to a decrease in viability. Our two parameter analysis of viability, based on intensity and size, allowed for a reliable calculation of live cells in a sample. This method of calculating viability of encapsulated cells using flow cytometry, has never before to our knowledge been designed. We have claimed the development of this assay and rationale behind the analysis, to be novel. It was used for the viability calculation in the remainder of the experiments.

3.2. Analysis of encapsulated G8 and BOEC cells secreting FVIII in vitro

G8 myoblasts and blood outgrowth endothelial cells (BOECs) have been studied for their ability to secrete therapeutic levels of FIX from capsules, and FVIII from a scaffold, respectively [7, 96]. The promising results achieved from studies using these cell lines for hemophilia gene therapy provided us with the incentive to study their behaviour in APA capsules, with respect to their secretion levels of FVIII, granularity and viability.

Granular cells, although unhealthy, may still be viable (explained in section 1.7.5). Granularity and viability of cells may change once encapsulated and can greatly influence the secretion ability of the cells. For the purposes of gene therapy, it was important to study how these two characteristics of cells can change once they are encapsulated and also to establish their effect on FVIII secretion. These different characteristics for encapsulated G8 and BOEC cells *in vitro* were examined, as well as for encapsulated G8 cells *in vivo*. Both cell lines were genetically modified to secrete canine FVIII. Viability was calculated by our method described in section 3.1.4.

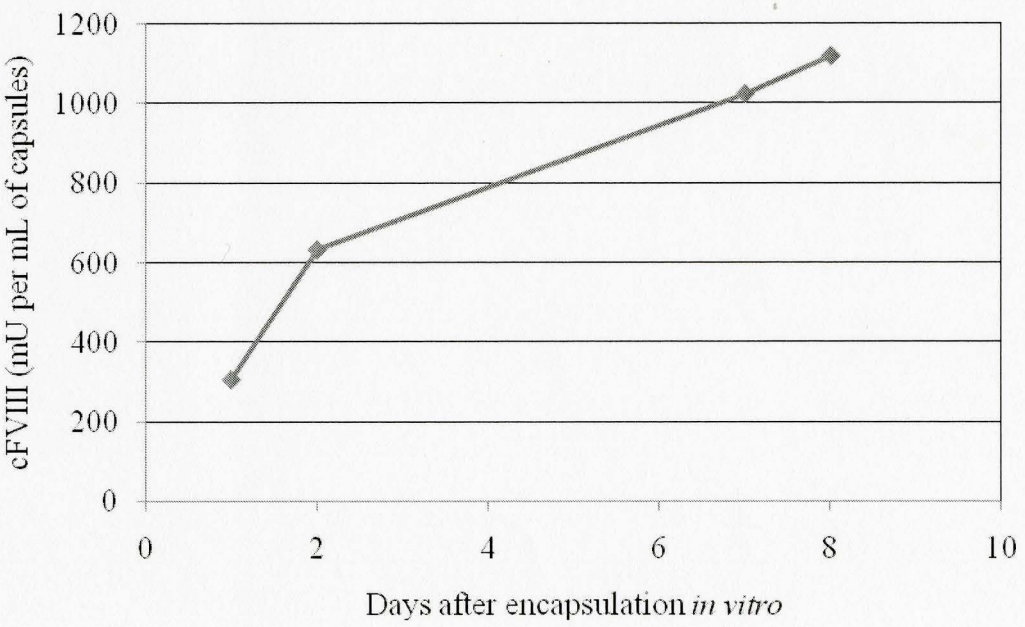
3.2.1. G8-cFVIII *in vitro*

3.2.1.1. Secretion from encapsulated G8 myoblasts *in vitro*

G8 myoblasts, transduced with a lentiviral vector encoding the canine FVIII transgene, were generously provided by Dr. Lillicrap's laboratory in Kingston, Ontario. The secretion of cFVIII from the non-encapsulated G8 cells was first assessed, prior to encapsulation. Over a 24 hour period, the free G8-cFVIII cells secreted 465.3 mU/ml of cFVIII (per $1x10^6$ cells) as measured by aPTT.

We proceeded to encapsulate these high secreting G8 myoblasts in APA capsules and measured their secretion levels for one week *in vitro.*

(A)



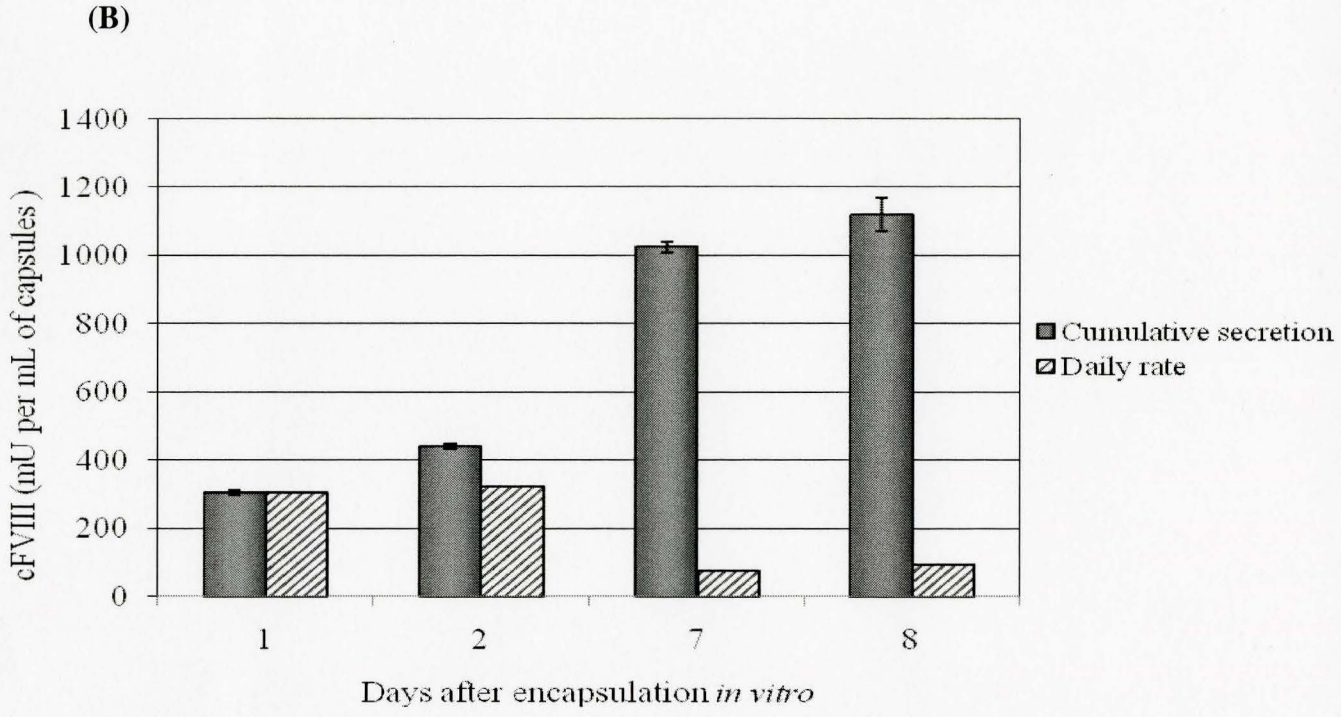


Figure 11 cFVIII levels from encapsulated G8 myoblasts cultured *in vitro.* Encapsulated G8 myoblasts secreting cFVIII were cultured *in vitro* and media samples were collected for 8 days to be assessed by ELISA (A) Cumulative cFVIII secretion showing increasing secretion over time (B) Total cFVIII secretion data plotted alongside daily rate of secretion as measured on that day. The graph indicates an accumulation of cFVIII from the capsules over time. Values Values represented as mean secretion (mU per ml of capsules) ± SD

Figure 11A shows that cFVIII release from the capsules steadily increases over the week, reaching approximately 1U secretion per ml of capsules. Each ml of capsules contained 3-4million cells, thus providing a 24-hour secretion of ~100mU of cFVIII per

$1x10^6$ cells, corresponding to 10% of normal canine FVIII activity. Figure 11B relates this cumulative secretion to the daily secretion rate as measured on the particular day. The daily rate of secretion, as indicated by the dashed series, remained consistent over days 1 and 2, but had however decreased on day 7. This drop in secretion could be attributable to a drop in viability of the encapsulated cells, which was further verified through flow analysis. Overall, the FVIII secretion from the G8 capsules remained high and reached therapeutic levels *in vitro* (>2 ng/ml).

3.2.1.2. Viability of encapsulated G8 myoblasts *in vitro*

The ability of the cells to secrete high levels of FVIII from the capsules *in vitro*, is likely to be correlated to their viability. However, secretion is also affected by granularity cellular health) and may not always change proportionally with viability. Thus, the viability and granularity of these G8-cFVIII myoblasts within capsules was studied and any changes over the week were observed.

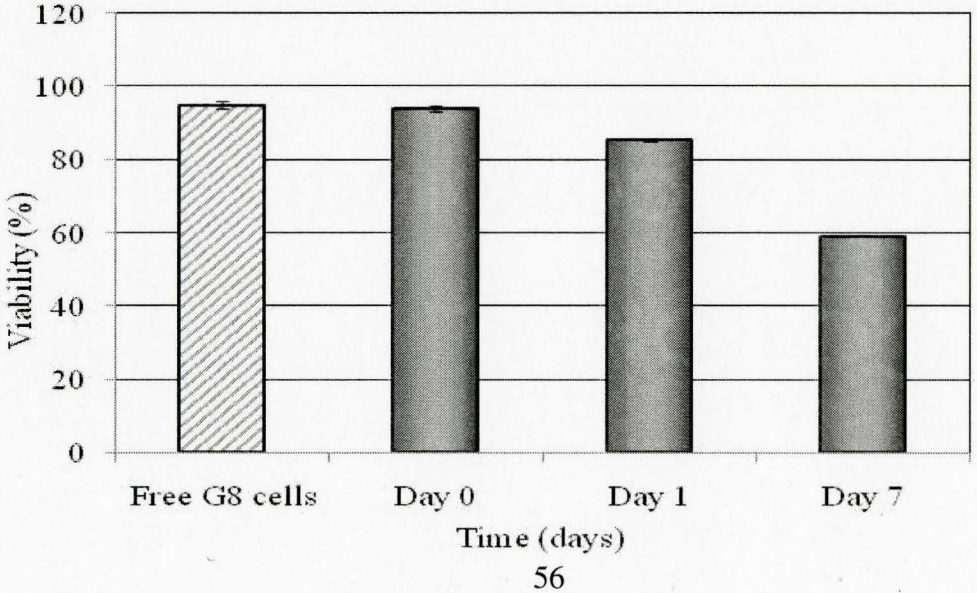


Figure 12 Viability of G8-cFVIII free (non-encapsulated) and encapsulated myoblasts *in vitro*. Capsules were cultured *in vitro* and cell viability was assessed on day 0, 1 and 7 by flow cytometry. Free G8 cell viability was measured on day 0, 1, and 7 and reported as an average. Values are represented as mean viability ± SD.

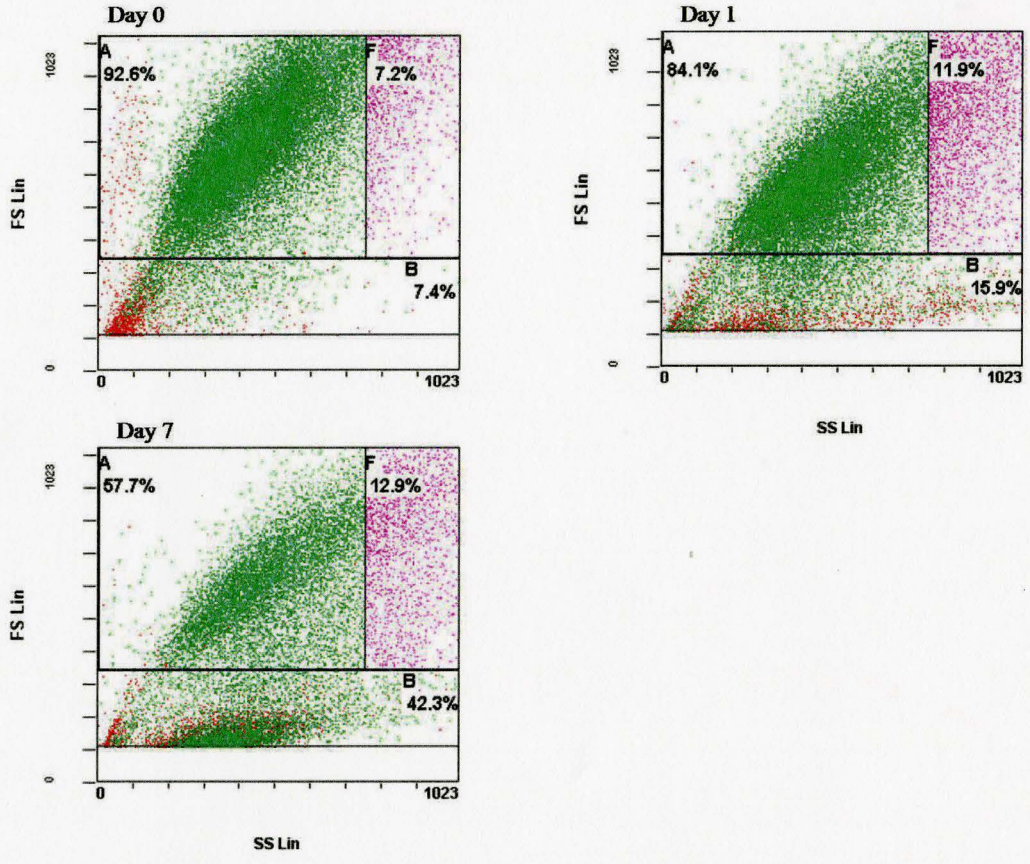


Figure 13 Flow cytometry (FF/SS plot) of encapsulated G8-cFVIII myoblasts cultured *in vitro*. Capsules were assessed on day 0, 1 and 7 after being made. Green = high intensity calcein labelled cells. Red = low/zero intensity calcein labelled cells. Box

A= cells >20μm, Box B = cells 5-20μm, Box F = subsection of Box A for granularity measurement

Figure 12 shows that the viability of cells following encapsulation, on Day 0 is high (93.88 ± 0.91%), with no significant difference from that of free G8 cells (94.9 ± 1.15%). The corresponding FS/SS flow plot for viability (Figure 13) on Day 0 shows the majority of cells that are brightly labelled with calcein (green) appear in the healthy, live Box A (as defined in section 3.1). Over the course of 7 days, a fraction of cells deteriorate in health as indicated by the movement of the calcein labelled cells (green) downwards into the unhealthy, dead Box B. Correlating this viability data with the cFVIII secretion data from Figure 11B, provides an explanation for the observed decrease in daily secretion rate on Day 7, as the viability of encapsulated cells also decreases on this day.

3.2.1.3. Granularity of encapsulated myoblasts *in vitro*

The degree to which the cells internal composition changes once encapsulated was also examined. The granularity of the free cells was fixed at 5.0% (Figure 9, panel II) and any deviation from this percentage in Box F of the sample was recorded as a change in granularity. The granularity of encapsulated cells on Day 0 increased from 5.0% to 7.2%. Furthermore, as the week progressed, more and more of the encapsulated cells became granular as indicated by the degree of side-scattering of all the cells. The group of live cells (green) observed on Day 0 no longer remained as a tight-knit cluster on Day 7

(Figure 13). This result suggests that once cells are enclosed in capsules, they immediately begin to vesiculate particulate matter, perhaps the alginate itself.

The results of G8 myoblasts capsules being cultured *in vitro*, indicate that the encapsulated cells were capable of secreting therapeutic levels of cFVIII, though the levels did not reach that of free (non-encapsulated) G8 cells. The viability data shows that myoblasts within capsules tend to decrease in viability over time, which can also be responsible for a drop in overall secretion. It also appears, from the granularity (side-scatter) measurement that the internal composition of the G8 cells starts to increase after being enclosed in capsules, and continues to do so over time.

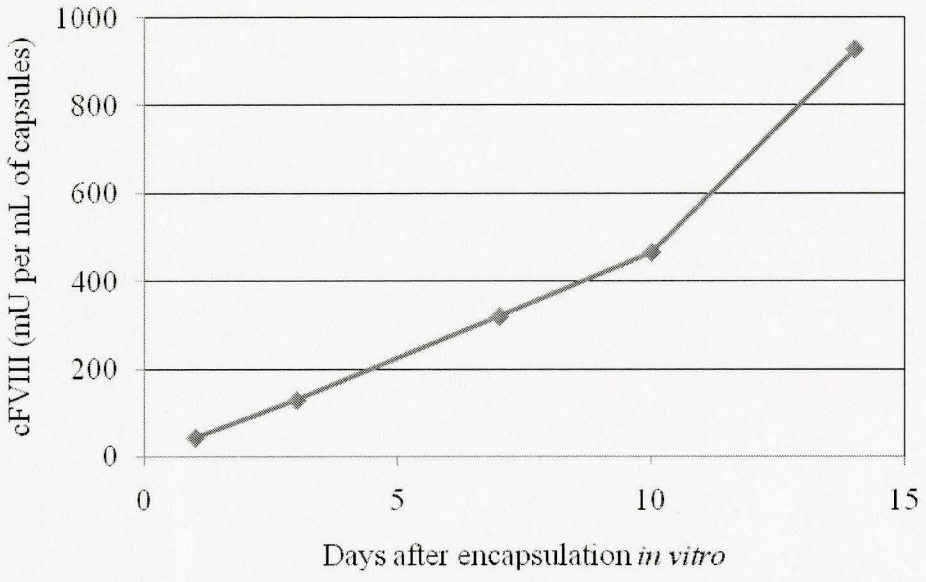
3.2.2. BOEC – cFVIII *in vitro*

To determine whether the effect of secretion, granularity and viability on encapsulated cells was cell line dependent, the behaviour of BOECs cells within APA capsules was also studied. A similar analysis to that of G8 myoblasts was performed. The cFVIII transduced BOECs, as with the myoblasts, were also generously provided to us by Dr. Lillicrap's laboratory in Kingston, Ontario.

3.2.2.1. Secretion from encapsulated BOECs *in vitro*

The 24-hour secretion of cFVIII from non-encapsulated BOECs was first assessed and found to be approximately 465.3mU/ml (per $1x10^6$ cells) as measured by aPTT The cells were then encapsulated in APA capsules and FVIII secretion was measured for 2 weeks.

(A)


1000
800
600
400
200
0
cFVIII (mU per mL of capsules)
0
5
10
15
Days after encapsulation *in vitro*

(B)

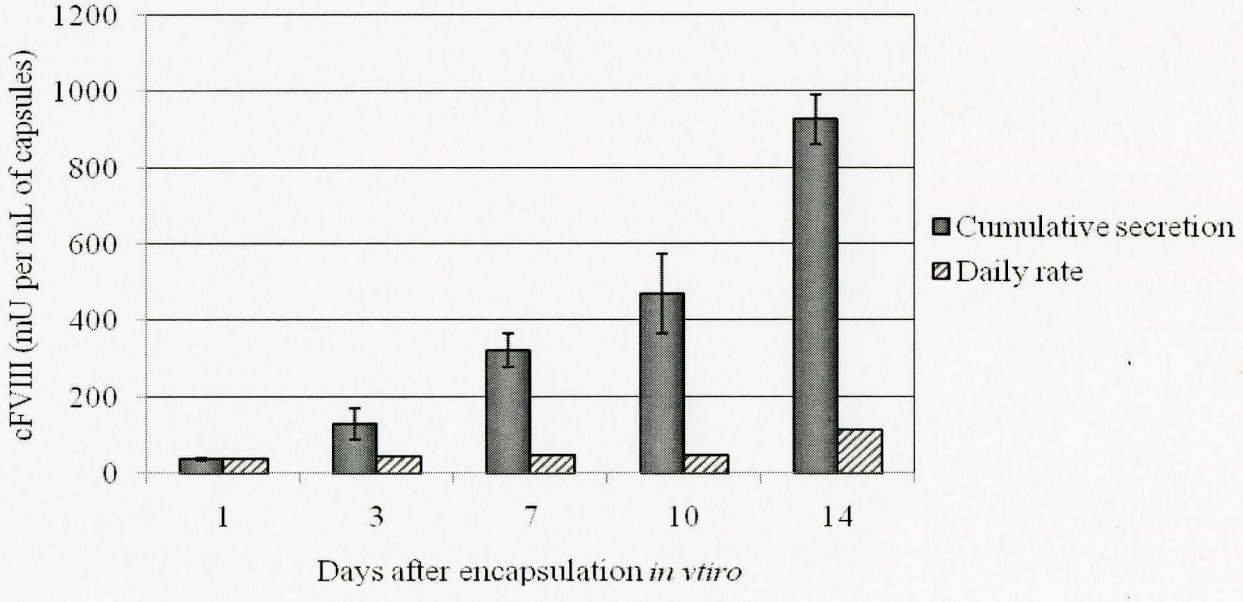

1200
1000
800
600
400
200
0
cFVIII (mU per mL of capsules)
1
3
7
10
14
Days after encapsulation *in vtiro*
Cumulative secretion
Daily rate

Figure 14 cFVIII levels from encapsulated BOEC myoblasts cultured *in vitro*. Encapsulated BOECs secreting cFVIII were cultured *in vitro* and media samples were collected for 14 days to be assessed by ELISA (A) Cumulative cFVIII levels showing increasing secretion over time (B) Total cFVIII secretion data plotted alongside daily rate of secretion as measured on that day. The graph indicates an accumulation of cFVIII from the capsules over time. Values represented as mean secretion (mU per ml of capsules) ± SD.

Figure 14A shows that cFVIII release from the capsules steadily increases, reaching approximately 1U per ml of capsules by the end of the second week. In comparison to the encapsulated G8 myoblasts, the encapsulated BOECs require approximately twice as much time to reach the same FVIII secretion level, suggesting that perhaps this cell line is not as efficient (slower rate) at producing the protein as G8 myoblasts. Indeed, this is confirmed with the daily rate of secretion as shown by the dashed series in figure 14B. The absolute rate of secretion on day 1 and day 7 of the BOECs is lower than that of the G8 myoblasts on the same day (Figures 11B and 14B). However, although the rate is lower, it is maintained at the same level for majority of the two weeks. The rate of secretion was likely to be influenced by cellular health; hence we proceeded to measure viability and granularity of the encapsulated BOECs to determine a correlation. Overall, the FVIII secretion from the BOEC-capsules remained high and reached therapeutic levels *in vitro* (>2 ng/ml).

3.2.2.2. Viability of encapsulated BOECs *in vitro*

As with the encapsulated G8 myoblasts, the viability and granularity for BOECs within capsules was also examined. The change in these factors changed as a result of encapsulation were studied.

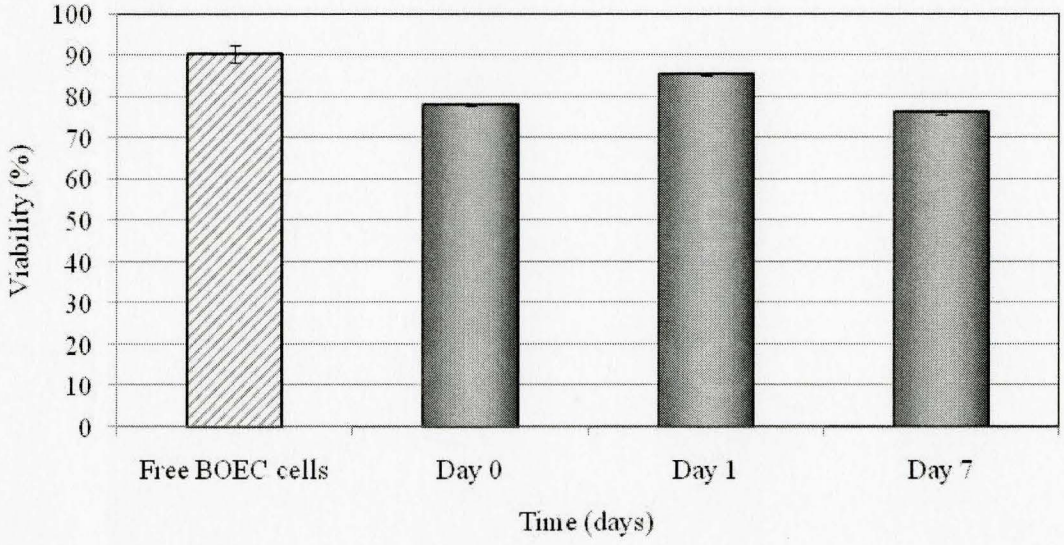


Figure 15 Viability of free (non-encapsulated) and encapsulated cFVIII-BOECs. Capsules were cultured *in vitro* and cell viability was assessed on day 0, 1 and 7 by flow cytometry. Values are represented as mean viability ± SD.

The viability of BOECs in the capsules was expected to be similar to that of free cells (non-encapuslated), on the day of encapsulation (day 0). Figure 15 shows that the viability of encapsulated BOECs on day 0 was 77.98 ± 0.18% (as calculated by equaltion 1). This drop in viability from free cells (90.3 ± 2.10%) may have been due to poor encapsulation conditions or negative response of the BOECs to the new environment as

they have never before been encapsulated. Over time, however, the viability does not fluctuate and stabilizes by day 7. This stabilization is better visualized on the flow cytometery FS/SS plot (Figure 16).

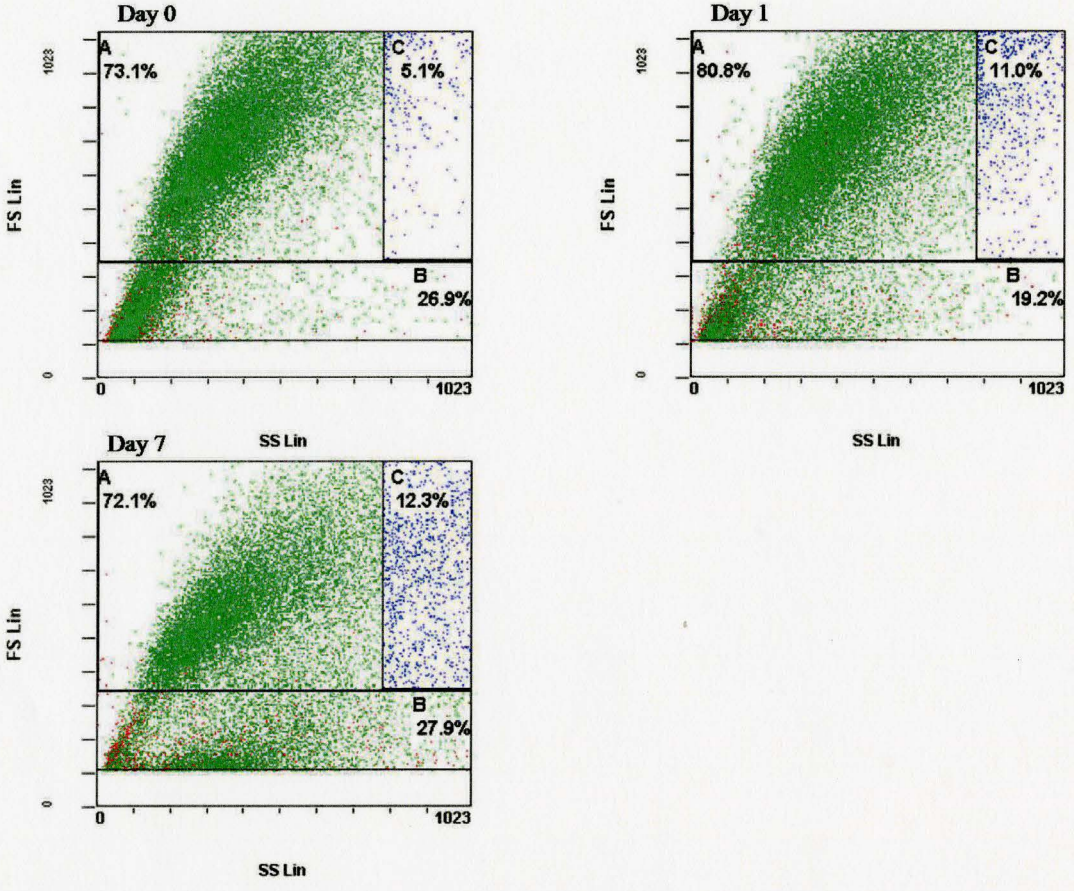


Figure 16 Flow cytometry (FF/SS plot) of encapsulated BOEC-cFVIII myoblasts cultured *in vitro*. Capsules were assessed on day 0, 1 and 7 after being made. Green = high intensity calcein labelled cells. Red = low/zero intensity calcein labelled cells. Box A= cells >20μm, Box B = cells 5-20μm, Box F = subsection of Box A for granularity measurement.

Majority of cells that are brightly labelled with calcein (green), cluster in box A, indicating they are alive and healthy. There is little movement of these live cells over the course of the week, with a relatively constant proportion remaining in box A. This stabilization of viability of BOECs within the capsules also correlates with the secretion data from Figure 14B. The daily rate of secretion (dashed series) remains comparatively stable. Secretion is often a direct result of how viable the cells are, but can also be affected by granularity.

3.2.2.3. Granularity of encapsulated BOECs *in vitro*

The granularity for the BOEC free cells was fixed at 8.0% (not shown) and any deviation from this percentage in Box F of the sample was recorded as a change in granularity. Much like the G8 myoblasts, the granularity of encapsulated BOEC cells increased over the week, from 8.0% to 12.3% on day 7. The BOEC cells, however, did not increase in side scatter to the same degree as encapsulated myoblasts as there was less spread among the live cluster. It would appear, from the consistency in FVIII secretion rates, stable viability and lower spread of BOEC cells, that this cell line adjusts better to capsular environment than G8 cells, if viability analysis using flow cytometry is correct.

3.2.3. Summary of encapsulated cell lines secreting cFVIII *in vitro*

The results from the *in vitro* study of G8 and BOEC encapsulated cells confirmed that both cell lines were capable of continous secretion of cFVIII from the capsules. Garcia *et al* (2002) reported similar findings when they measured the *in vitro* secretion of cFVIII from both free and encapsulated C2C12 cells. The viability of encapsulated G8

myoblasts on day 0 (~93%) was found to be higher than that of other encapsulated cell viability studies reported by Garcia *et al* (2002), and Wen *et al* (2007). The viability of encapsulated BOECs was also remained high, averaging ~80% over the course of 7 days, and mirrored the steady rate of cFVIII from the capsules. The development of the flow cytometric method of viability measurement ('calcein loss') allowed for reporting cell viability with a higher degree of accuracy verses current studies which utilize trypan blue exclusion. Common to both cell lines was the increase in granularity after encapsulation. Overall, the *in vitro* study of both cell lines revealed that G8 myoblasts and blood outgrowth endothelial cells were capable of continuous cFVIII release from capsules, while sustaining high viability over one week. The results of our *in vitro* study on encapsulated myoblasts and BOECs, support their suitability as a cell line for cFVIII delivery *in vivo.*

3.3. Analysis of Factor VIII secreting encapsulated G8 myoblasts in vivo

The *in vitro* study established that encapsulated G8 myoblasts were capable of maintaining high cell viability and secreting cFVIII above therapeutic levels, and thus we proceeded to evaluate their effectiveness *in vivo.* The same factors - secretion, viability, granularity - were examined and used to assess whether the G8 microcapsules could be an effective treatment for cFVIII delivery in a murine model.

3.3.1. Implantation of C57BL/6 immunocompetent mice with encapsulated G8 myoblasts secreting cFVIII

3.3.1.1. Secretion of cFVIII *in vivo*

The goal of this *in vivo* study was to enclose G8 myoblasts in APA capsules and measure cFVIII levels after implantation into normal mice. The feasibility of using capsules *in vivo* was first assessed in normal mice before testing them in FVIII-deficient KO mice (hemophilic). A total of $20x10^6$ encapsulated cells ($5x10^6$ cells per ml of capsules) was implanted intraperitoneally (IP) into immunocompetent C57BL/6 mice. The mice were bled prior to implantation, on day 1 post-implantation, and at weekly intervals thereafter, and the levels of cFVIII in the plasma were quantified by ELISA.

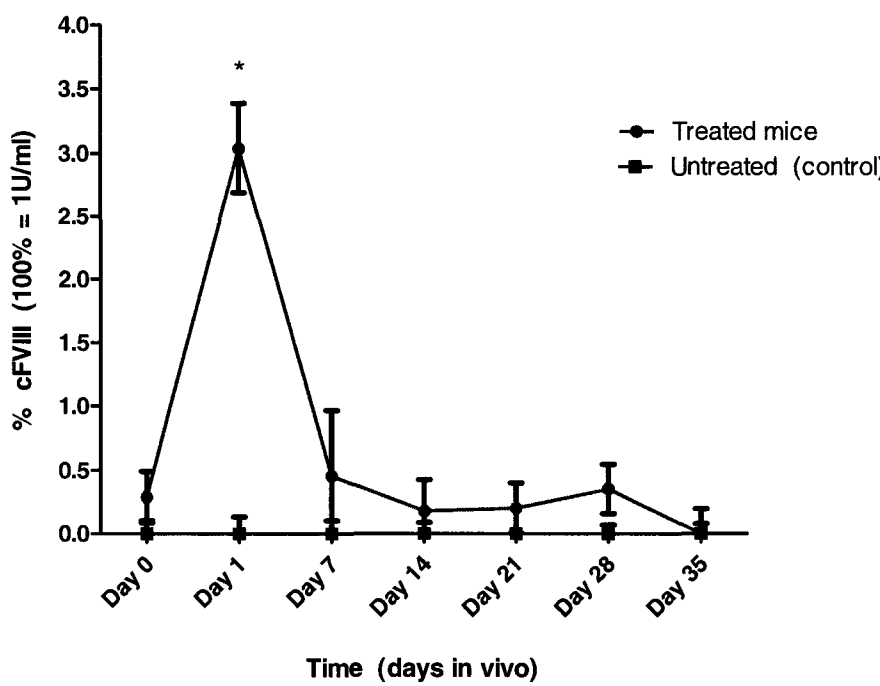


Figure 17 Average plasma levels of cFVIII in C57BL/6 mice. Normal mice (n=4) were implanted with encapsulated G8 myoblasts secreting cFVIII. Normal mice (n=4) that did not receive capsules were used as control. Blood samples from treated mice were collected at various time points and plasma was assessed by ELISA. The graph shows cFVIII levels for treatment mice (n=4) on day 1 are extremely significant when compared to treatment mice on day 0 (pre-implantation). Values are represented as mean cFVIII levels ± SD. * $p < 0.001$

Figure 17 shows the average cFVIII levels in normal mice at weekly intervals after implantation. Up to 35.6 ± 7.0 mU/ml of cFVIII was detected in the plasma of treated mice on day 1, corresponding to approximately 3.5% of normal levels. This increase in cFVIII levels on day 1 in the treatment mice was statistically significant when compared to treatment mice on day 0 ($p<0.001$). However, the delivery of cFVIII was transient in all mice and reached the maximum concentration on day 1, decreasing thereafter. To determine whether this transient secretion from the encapsulated cells could be recovered *in vitro*, mice were sacrificed and the capsules were retrieved (5 weeks post-implantation).

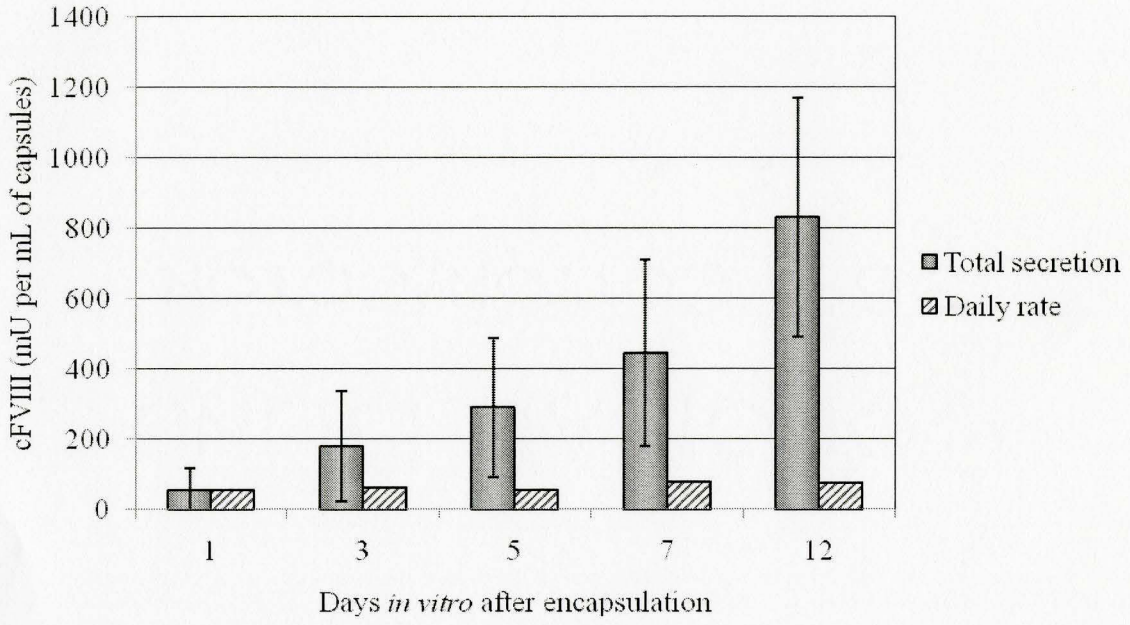


Figure 18 cFVIII levels from encapsulated G8 myoblasts retrieved from C57BL/6 mice. Capsules were cultured *in vitro* upon retrieval and media samples were collected for 12 days to be assessed by ELISA. Values represented as mean secretion (mU per ml of capsules) ± SD.

The retrieved capsules were cultured *in vitro* for up to 12 days and cFVIII levels were measured at specific time points. The G8 cells were capable of steady cFVIII release from the capsules, maintaining a consistent rate of secretion over time as shown in Figure 18. The secretion from the encapsulated cells upon retrieval on day 0 was approximately 6 times less than secretion levels from non-implanted capsules cultured solely *in vitro* (3.2.1.1, Figure 11). By day 7, the secretion rate is close to double that of day 1, suggesting an improvement in the relative health of the cells. To confirm this, we measured the cell viability upon retrieval from the normal mice.

3.3.1.2. Viability of encapsulated myoblasts retrieved from C57BL/6 mice

The viability of the encapsulated cells retrieved from normal mice was assessed using the flow cytometry assay developed in Section 3.1. Based on the decline in cFVIII levels in the mouse after day 7 (Figure 17) the viability upon retrieval was expected to be low.

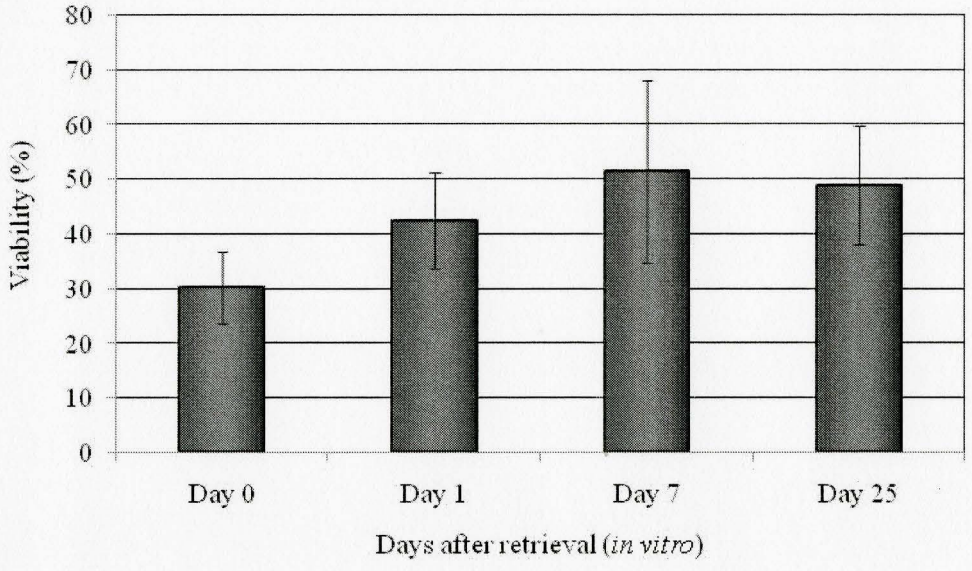


Figure 19 Viability of encapsulated G8-cFVIII myoblasts retrieved from normal C57BL/6 treated mice. Capsules were retrieved 5 weeks after implantation and cultured *in vitro*. Cell viability was assessed on day 0, 1, 7 and 25 after retrieval, by flow cytometry. Values are represented as mean viability ± SD.

Figure 19 shows that immediately after being retrieved from the mice on day 0, the average encapsulated cell viability was low at 30.2 ± 6.6 %. However, when cultured in optimal cell culture conditions, the cells showed rapid improvement and recovery in

viability. By day 7, the calculated viability rose by approximately 20%. The improvement in viability was observed close to one month, and by day 25 the viability had approached pre-implantation levels similar to that of *in vitro* G8-cF8 capsules. The recovery in cell viability after retrieval can be visualized from the representative flow cytometry plot of mouse 1, whose viability reached 72.1% after one week.

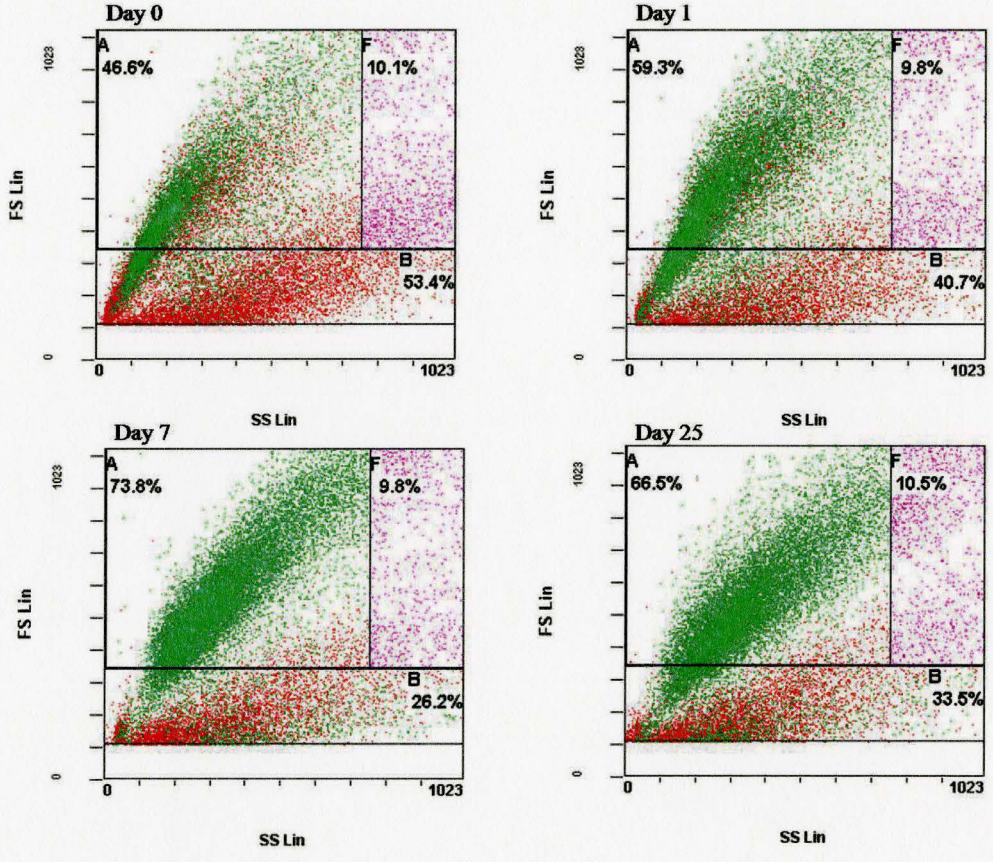


Figure 20 Representative flow cytometry (FF/SS) plot of encapsulated G8-cFVIII myoblasts retrieved from C57BL/6 mouse #1. Capsules were cultured *in vitro* and assessed on day 0, 1, 7 and 25 after retrieval. Green = high intensity calcein labelled cells.

Red = low/zero intensity calcein labelled cells. Box A= cells >20μm, Box B = cells 5-20μm, Box F = subsection of Box A for granularity measurement

The gradual improvement in viability can be visually observed from figure 20, as the healthy, live box A becomes populated by those cells that are expressing calcein at high intensities (green). By day 7, majority of the large unhealthy cells have died off and receded into box B (red), while the tight knit cluster of live cells has clearly defined itself as healthy in box A. The improvement in cellular viability of the retrieved capsules is reflected in the increase in secretion rate, also on day 7 (Figure 18). Secretion levels however did not reach the levels achieved by G8-cF8 capsules *in vitro,* suggesting that although there was recovery in the viability of encapsulated G8-cF8 cells after retrieval, a proportion of viable cells may be still be unhealthy thereby preventing an increase in secretion to the same degree. The increase in granularity upon retrieval on day 0, compared to free cells (5.0% to 10.1%, Fig 7 and 18 respectively) may be representative of an unhealthy proportion of cells that were not able to secrete cFVIII as efficiently as the healthy fraction.

3.3.2. Implantation of C57BL/6 Hemophilia A (FVII-deficient) KO mice with encapsulated G8 myoblasts secreting cFVIII

3.3.2.1. Secretion of cFVIII *in vivo*

The goal of this study was to implant G8-cF8 capsules into hemophilia A mice and assess the *in vivo* levels of cFVIII secreted by the encapsulated cells. A total of $20x10^6$ encapsulated cells ($5x10^6$ cells per ml of capsules) were implanted intraperitoneally (IP) into immuno-competent hemophilia A mice. The mice were bled prior to implantation, on day 1 post-implantation, and at weekly intervals thereafter, and the levels of cFVIII in the plasma were quantified by ELISA.

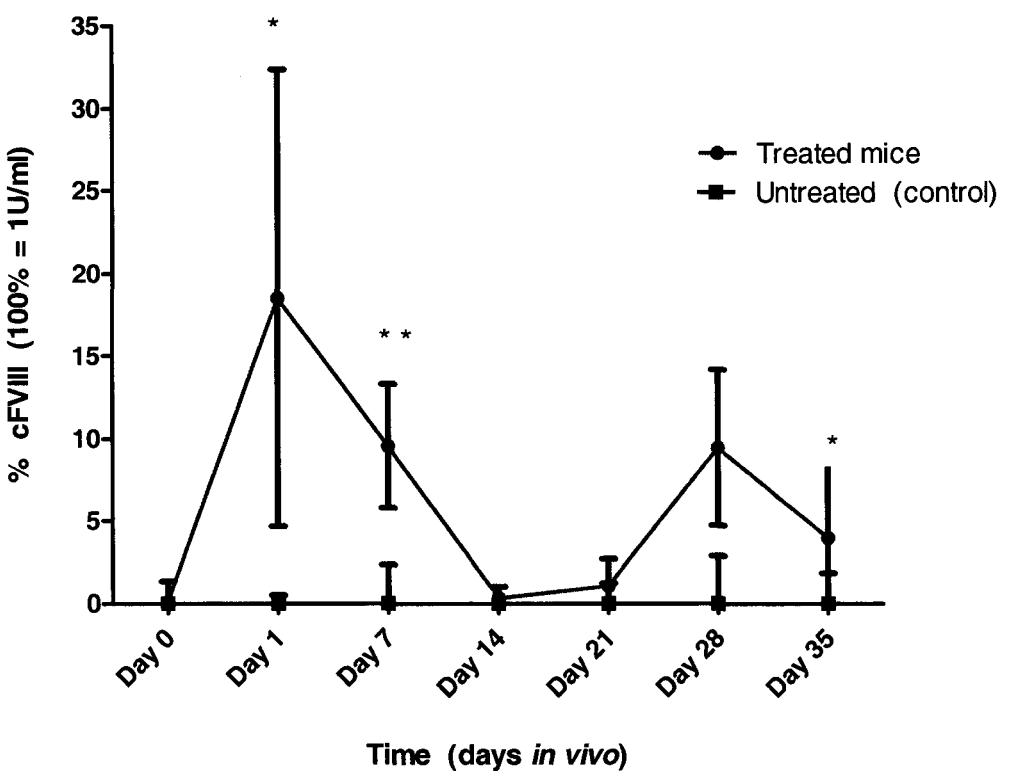


Figure 21 Average plasma levels of cFVIII in hemophilia A mice. Hemophilic mice (n=4) were implanted with encapsulated myoblasts secreting cFVIII. Hemophilic mice (n=3) that did not receive capsules were used as control. Blood samples from treated mice were collected at various time points and plasma was assessed by ELISA. The graph shows cFVIII levels for treated mice (n=4) on days 1, 7 and 35 were statistically significant when compared to treated mice on day 0 (pre-implantation). Values are represented as mean cFVIII levels ± SD * < 0.05, ** $p < 0.001$

Figure 21 shows the average cFVIII levels in hemophilia A mice at weekly intervals after implantation. The maximum cFVIII concentration in the plasma of treated mice was observed on day 1, reaching a level of 18.5 ± 5.3 % of normal levels. High plasma cFVIII levels persisted until day 7, at 9.5 ± 3.8%. The day 1 and day 7 plasma levels for treated mice showed a statistically significant increase from their day 0 plasma levels (before treatment). However, by day 14 the cFVIII concentration had decreased close to pre-treatment plasma levels. Starting from day 28, the presence of cFVIII was detected in the plasma of 50% of the treated mice.

Five weeks after implantation, the capsules were retrieved from the treated mice and cultured *in vitro*. The encapsulated myoblasts were capable of continuous cFVIII secretion over time, but at a decreased rate from that of non-implanted capsules *in vitro* (data not shown).

3.3.2.2. Viability of encapsulated myoblasts retrieved from Hemophilia A mice

The viability of retrieved encapsulated cells from hemophilic mice was assessed using the flow cytometry assay developed in Section 3.1.

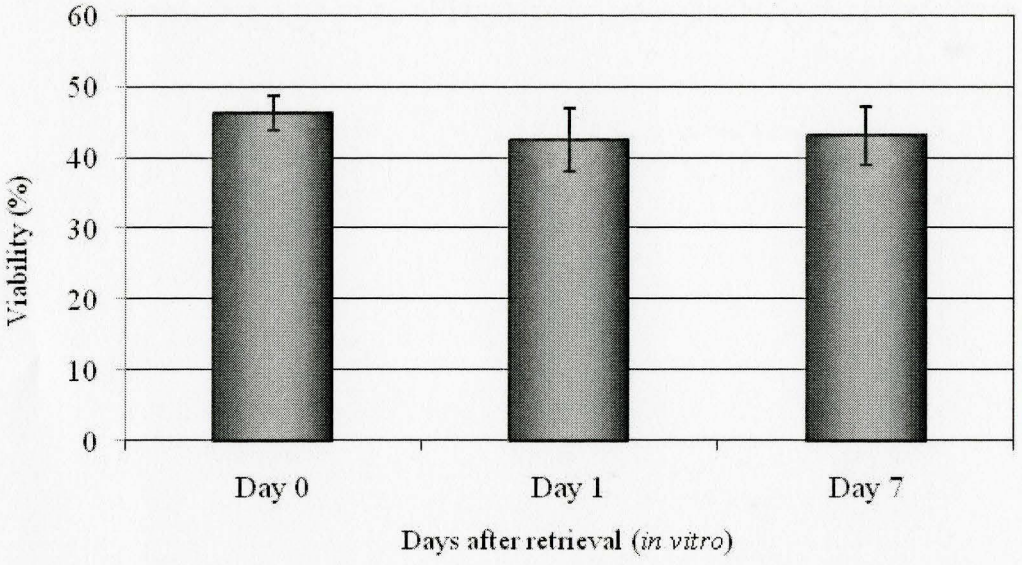


Figure 22 Viability of encapsulated G8-cFVIII myoblasts retrieved from hemophilia A treated mice. Capsules were retrieved 5 weeks after implantation and cultured *in vitro*. Cell viability was assessed on day 0, 1, and 7 after retrieval, by flow cytometry. Values are represented as mean viability ± SD.

Figure 22 shows that upon retrieval from hemophilic mice on day 0, the encapsulated cell viability was 46.2 ± 2.4%. The viability remained relatively unchanged throughout the week, stabilizing at 43.1± 4.4% by day 7. The constancy in viability can be visualized by a representative flow cytometry plot for hemophilic mouse 1,

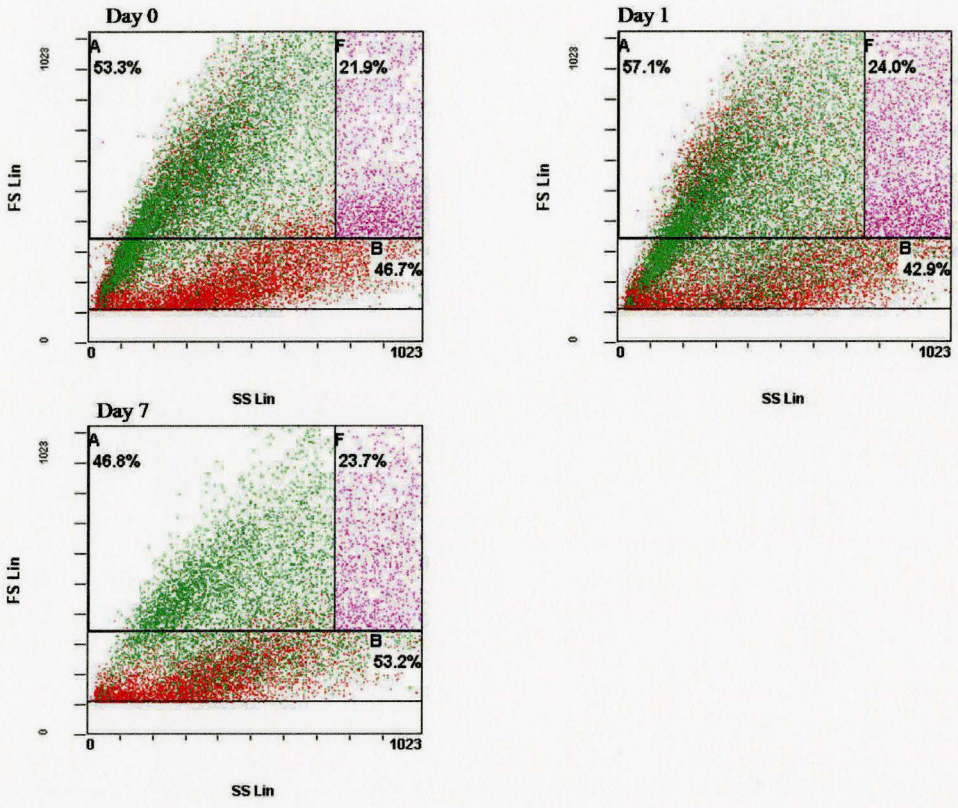


Figure 23 Representative flow cytometry (FF/SS) plot of encapsulated G8-cFVIII myoblasts retrieved from hemophilic mouse #1. Capsules were cultured *in vitro* and assessed on day 0, 1 and 7 after retrieval. Green = high intensity calcein labelled cells. Red = low/zero intensity calcein labelled cells. Box A= cells >20μm, Box B = cells 5-20μm, Box F = subsection of Box A for granularity measurement

The stabilization in viability of the retrieved encapsulated cells can be visually observed from Figure 23. The proportion of live cells in box A that are intensely labeled with calcein (green) remains steady over the week. The granularity of the retrieved cells from hemophilic mice on day 0, 1 and 7 show a statistically significant increase ($p < 0.01$)

from that of non-implanted G8 capsules cultured *in vitro* on the same days (Figures 13 and 23, Box F). This increase in internal cell composition is best visualized by comparing the degree of side-scatter in from encapsulated cells *in vitro* (Figure 23) verses retrieved encapsulated cells (Figure 13). The granularity increase suggests a change in cell morphology as a result of capsules being *in vivo* for 5 weeks.

3.3.2.3. Biological Activity of cFVIII delivered in Hemophilia A mice: aPTT assay

The cFVIII ELISA (from 3.3.2.1) showed therapeutic levels of cFVIII being delivered in the hemophilic mice treated with the encapsulated myoblasts. In order to determine whether the cFVIII detected by ELISA was also biologically active, a plasma aPTT assay was performed on the treated mice and assessed function based on clotting time.

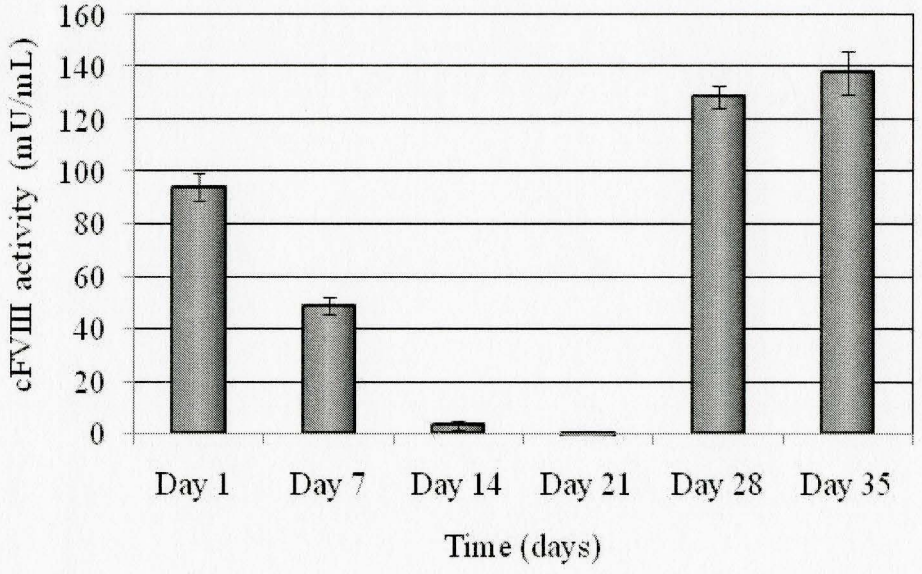


Figure 24 Activated partial thromboplastin time (aPTT) of plasma from treated hemophilia A mice. Hemophilic mice (n=4) were implanted with encapsulated G8 myoblasts secreting cFVIII. Mice were bled at weekly intervals and plasma was assayed for the presence of functional cFVIII. Normal canine plasma was used to generate a standard curve for determining cFVIII activity in samples. Values represented as mean clotting time ± SD.

Figure 24 shows that cFVIII in the plasma of treated mice, showed a statistically significant increase in activity on days 1, 7, and 35, as measured by aPTT assay. The cFVIII activity declined on days 14 and 21. The pattern of cFVIII activity observed with aPTT, correlate with the cFVIII concentration measured on each day by ELISA in section 3.3.2.1. Thus, the encapsulated myoblasts were capable of delivering functional cFVIII in hemophilic mice.

The plasma from treated (implanted with G8-cFVIII capsules) and control (non-implanted) hemophilic mice was assayed for the presence of anti-cFVIII antibodies, by ELISA, on day 42. Plasma from normal C57BL/6 mice that were immunized with canine plasma, was also assayed for comparison.

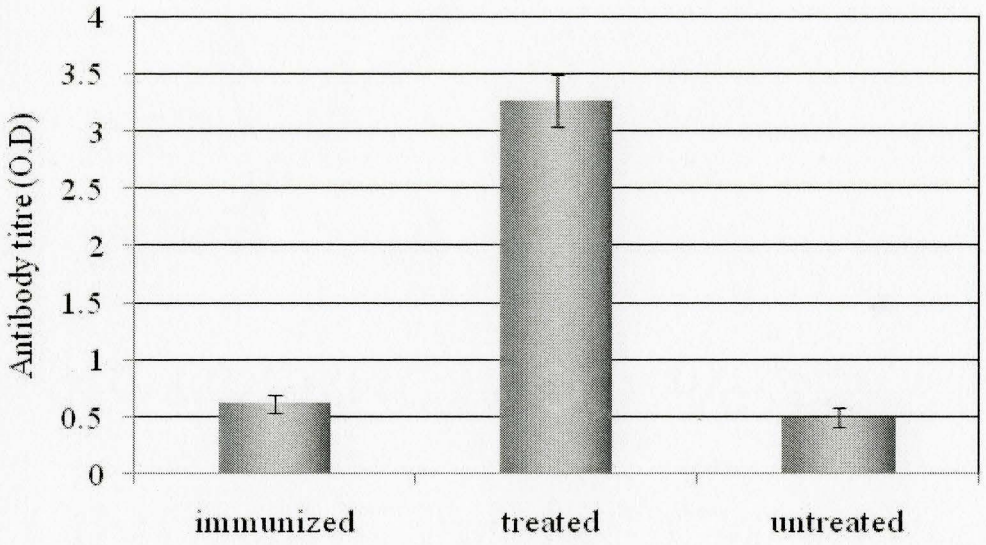


Figure 25 Development of anti-cFVIII antibodies in implanted hemophilic mice. Anti-cFVIII antibodies in mice (n=4) implanted with encapsulated G8 myoblasts were quantified by ELISA. A group of untreated hemophilic mice (n=3) that did not receive capsules were used as control. Another group of normal mice (n=3) immunized with canine plasma were included as a positive control. High antibody titres were found in all of the treated mice (implanted with capsules).

A vigorous titre of antibodies was detected in all of the mice that were implanted with encapsulated myoblasts secreting cFVIII, in contrast to the levels of antibodies detected in control mice. Immunized mice had higher titre than controls, but far lower than implanted mice.

3.3.2.4. Delivered cFVIII offered protection to Hemophilia A mice against trauma: Tail-clip test

The ultimate goal of any hemophilia treatment is to offer protection against excessive bleeding after trauma. Thus, a standardized tail-clip assay was performed on both untreated hemophilic mice and hemophilic mice treated with the cFVIII secreting encapsulated G8 myoblasts, and assessed their ability to survive this serious injury. The tail-clip assay was performed on day 7 after implantation of capsules, as therapeutic levels of cFVIII was shown to be detected on this day (confirmed by ELISA, 3.3.2.1). Two days after the tail cut, all of the untreated mice appeared sick, as they were hunched, cold to touch, shivering and immobile in one corner of the cage. Upon request from the veterinary staff of the central animal facilities (C.A.F, McMaster University), three days after the tail clip, the untreated control mice were sacrificed. In contrast, the treated mice appeared active and healthy. Blood samples were taken from all of the mice and the hematocrit (packed red blood cells) was measured as a percentage of total volume.

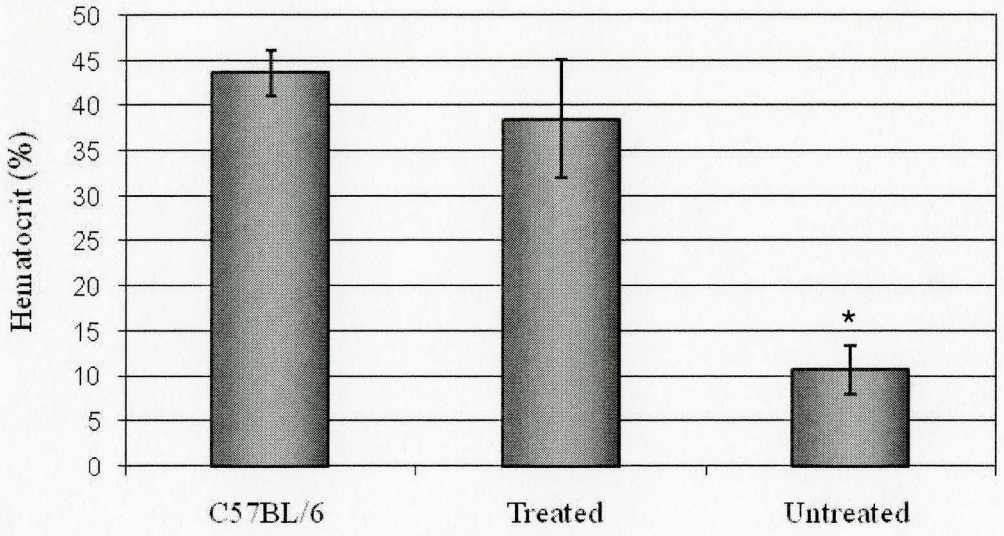


Figure 26 Percentage of hematocrit in normal, hemophilic treated and hemophilic untreated mice. Hemophilic mice (n=3) were implanted with encapsulated G8 myoblasts secreting cFVIII. Hemophilic mice (n=3) that did not receive capsules were used as control. Normal C57BL/6 mice (n=3) were used as a positive control. Tail-clip was performed on day 7 after implantation. Blood samples were collected 3 days post-tail clip and hematocrit levels were measured. * $p < 0.01$ when comparing untreated to treated mice. * < 0.001 when comparing untreated to normal mice. No significant difference was found between treated mice and normal C57BL/6 mice. Values are represented as mean hematocrit ± SD.

As shown in Figure 26, the untreated mice had a statistically significant decrease in the percentage of hematocrit, (10.8 ± 2.7%) than the hemophilic mice implanted with G8-cFVIII capsules, (38.5 ± 6.5%). The difference in percentage of red blood cell

volume between normal, treated and untreated mice was indicative of their health status. The hematocrit percentage of the untreated mice confirmed their anemia due to extensive blood loss. 100% of the treated mice survived (with no statistically significant reduction in hematocrit), suggesting that the encapsulated myoblasts provided mice with enough cFVIII to offer protection against blood loss in a model of major trauma. There was no statistical significance between treated and normal C57BL/6 mice, but untreated mice had a significant reduction in hematocrit ($p< 0.01$, $p<0.001$) compared to treated and normal C57BL/6 mice, respectively.

3.3.3. Summary of *in vivo* experiments delivering encapsulated G8 myoblasts secreting cFVIII

The results of the *in vivo* experiments showed that encapsulated G8 myoblasts are capable of delivering therapeutic levels (> 1% of normal) of cFVIII in both normal and hemophilic mice. Both experiments showed statistically significant increases of cFVIII on day 1, when compared to day 0 levels. The clearance of cFVIII by day 7 in the plasma of normal C57BL/6 mice may be in part due to loss of viability as observed upon retrieval. In contrast, viability of encapsulated myoblasts from hemophilic mice was approximately 20% higher upon retrieval, resulting in persistent expression of cFVIII *in vivo*. Our day 1 levels of cFVIII in hemophilic mice (180mU/ml, or ~18% of normal levels) were comparable to the study by Garcia *et al* (2002) that also reported hFVIII concentration, (delivered by encapsulated C2C12 cells) to be highest on day 1 (22ng/ml or ~10-20% of normal levels) [7]. The comparison is complicated by the fact that different species

versions of FVIII were used in our respective studies. The findings of Garcia *et al* (2002) showed that hFVIII levels continually decreased after day 1 until it disappeared completely by day 7. They also found that the transient delivery in all the mice was accompanied by an increasing titre of antibodies. In contrast, in our study we found that therapeutic levels of cFVIII were able to persist in hemophilic mice until day 7, (~9.5% of normal levels), and decreased thereafter on days 14 and 21. High circulating cFVIII levels returned once again on days 28 and 35. The high cFVIII concentration on days 1, 7, 28 and 35 also corresponded to an increase in activity on the same days as measured by aPTT (clotting time). A similar pattern of transient decrease in cFVIII concentration (on day 14) and subsequent rise again, was observed by Wen *et al* (2006) in the study using encapsulated myoblasts secreting the FIX antigen [8, 9]. While it is difficult to reconcile the simultaneous presence of antigen and antibodies, a protection test performed on day 28-35 would be required to confirm or refute the persistence of functional FVIII.

The presence of antibodies at the end of the experiment (on day 35) was investigated, and found that there were detectable antibodies present in the plasma of all the treatment mice. However, our antibody titre, if expressed as a ratio of OD value ($OD_{day\ x}/OD_{day\ 0}$), was lower than the levels found by Garcia *et al* (2002), ~6 vs. ~12 respectively. If confirmed, this may be attributable to G8 myoblasts being a less immunogenic cell line than C2C12s; though these arbitrary ratios are difficult to compare in different ELISA tests. It is difficult to reconcile the presence of cFVIII antigen at the end of the experiment with the detection of antibodies, however only 50% of the treated

mice (2/4) had cFVIII levels return on day 35, while 50% showed complete disappearance of the protein.

It appears that the presence of antibodies found in the hemophilic mice did not affect the activity of cFVIII as verified by the aPTT results on day 28 and 35. We hypothesize that these antibodies were non-neutralizing and thus may not have had an inhibitory affect on the activity of cFVIII. However, to confirm the absence of inhibitors, a Bethesda assay will be performed on the plasma of treated mice. Non-neutralizing antibodies that target non-functional epitopes on FVIII might account for the detectable activity in the aPTT assay [102]. The possibility of transient inhibitors has been also recognized in the literature, in which inhibitors develop but 'spontaneously' regress with time despite continued intermittent exposure to FVIII [102-104].

Finally, the ultimate test for a hemophilia treatment is protection against excessive bleeding that may arise from trauma. Current studies have used the standardized tail-clip assay to assess protection in a qualitative fashion; by measuring the time it takes for treated mice to stop bleeding. In this study, a quantitative assessment of protection was employed by measuring hematocrit (percentage of packed red blood cells in total blood volume) in treated and untreated mice. Blood loss results in a reduction of the hematocrit, which can easily be quantified. Excessive blood loss can also lead to anemia, a condition where the number of healthy- oxygen carrying red blood cells is lower than normal [105]. Since hematocrit levels will decrease as a result of blood loss, their measurements are often used as diagnosis for anemia. Thus, hematocrit measurements

were used as an objective method for assessing the amount of blood loss that the mice had endured after trauma. The tail-clip was performed on day 7 after implantation with capsules, as the ELISA revealed that high levels of circulating cFVIII were still present on this day The untreated hemophilic mice displayed common signs of anemia including fatigue, weakness and shivering. Indeed, their levels of hematocrit were significantly lower than that of normal mice indicating blood loss, and as a result had to be euthanized upon request by veterinary staff. The hemophilic mice that were treated with G8-cFVIII capsules showed no signs of anemia and appeared to be healthy Their hematocrit levels showed no statistical difference compared with normal mice. The protection against bleeding in treated hemophilic mice was attributed to the presence of functional cFVIII. The protection of hemophilic mice against trauma is met with skepticism by the research community, due mainly to the subjective nature of the tail bleeding assay test. The objective measurement of the hematocrit may provide more validity to the test.

The study by Garcia *et al* (2002) reported the complete disappearance of hFVIII by day 7 After measuring a 5-fold reduction in FVIII mRNA levels from the C2C12 encapsulated cells, the decline in plasma FVIII levels was attributed to transcriptional repression *in vivo*. This transcriptional repression also resulted in a 30% decrease in hFVIII from the capsules upon retrieval. However, in our study it was found that, while FVIII levels delivered on day 1 were comparable to those found by Garcia et al (2002), high levels of cFVIII persisted until day 7 and decreased on day 14. Moreover, treated mice were protected on day 7 levels against blood loss after trauma, indicative of the

presence of biologically active cFVIII. The capsules were also able to continually secrete cFVIII upon retrieval from the mice, though at a rate less than that of capsules cultured *in vitro* prior to implantation. Thus, it is unlikely that transcription repression was the main cause for cFVIII decline.

Overall, our study overcomes one of the two main obstacles – disappearance of FVIII by day 7 and the formation of antibodies - faced by Garcia *et al* (2002) using encapsulated C2C12 cells. We report high FVIII levels that persist until day 7 and return again on day 28, an improvement over Garcia *et al*, whose cFVIII levels declined after day 1 Our choice of virus and cell type may have been contributing factors to the persistent expression that was observed. Lentiviral transduced cells appear to have a higher resistance against transgene inhibition verses the cells transduced with other retroviruses. Lentiviral infection has advantages over other gene therapy methods including high efficiency transduction of dividing and non-dividing cells, long term stable expression of a transgene, and low immunogenicity [106, 107] Also, transplanted fetal cells, like the G8 myoblasts, often do not trigger immune responses in a host, making them a more suitable cell line than the tumorgenic transformed C2C12 cell line [8, 78]

Although antibodies were also detected, the titre, if expressed as a ratio of OD, was lower than previous findings. The transcription repression that caused disappearance of FVIII in Garcia *et al* study, was an unlikely factor in our current work as cFVIII activity was sustained for at least one week after implantation, confirmed by aPTT and tail clip assay The treated mice were able to withstand blood loss after being subjected to

trauma, confirming the biological activity of circulating cFVIII. The presence of inhibitors still needs to be assessed by Bethesda assay, to elucidate whether the antibodies detected were non-neutralizing. This study shows that the implantation of encapsulated myoblasts secreting cFVIII delivered functional and therapeutic cFVIII levels days 1 and 7 If these levels of FVIII delivery were achieved in humans, it would be sufficient to convert a severe hemophiliac to a mild or moderate phenotype.

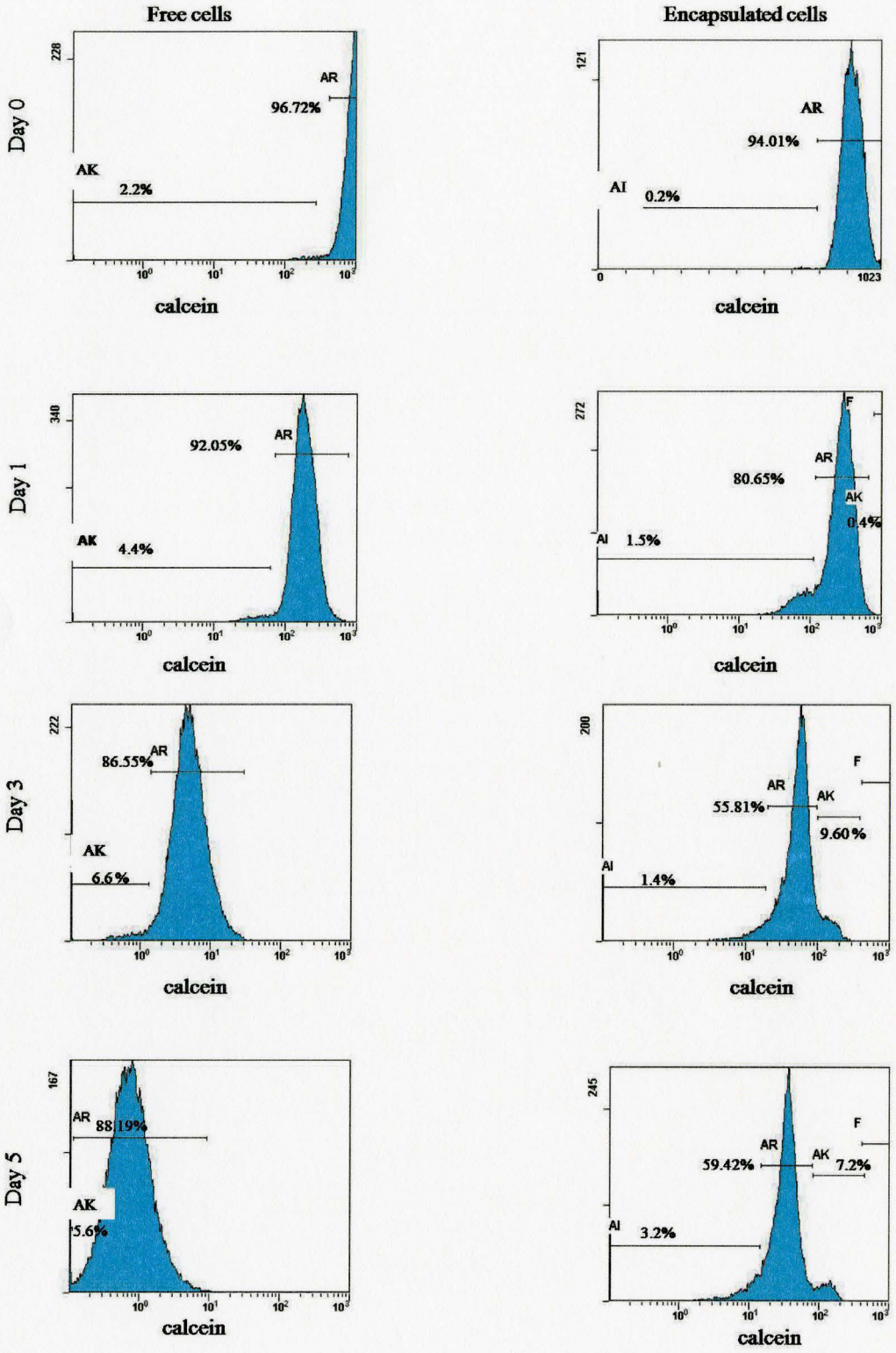
3.4. Flow cytometry based examination of encapsulated G8-cFVIII proliferation profile, using intracellular dye CFSE

Myoblasts are a suitable cell line to encapsulate since they have the advantage of terminally differentiating into myotubes. This characteristic of myoblasts ensures that they will not proliferate indefinitely within the microcapsules, thereby allowing the free diffusion of FVIII [88, 89] The actual proliferation profile of encapsulated myoblasts (the time-course breakdown of proliferative events), has never been examined. Encapsulated myoblasts may well differ from free (non-encapsulated) myoblasts with respect to their rate of proliferation, the proportion of cells that are able to undergo proliferation, and the proportion of dead cells that accumulate within the capsule. Thus, a novel flow cytometry based assay was developed to study the effect of encapsulation on the proliferative capacity of G8 myoblasts secreting cFVIII.

3.4.1. Proliferation prolife *in vitro*: encapsulated vs. non-encapsulated G8-cFVIII myoblasts

The goal of this study was to assess the proliferation profiles of free cells and encapsulated cells cultured *in vitro*, at various time intervals. G8 myoblasts secreting cFVIII were labeled with CFSE, (a fluorescent cell staining dye that is retained within cells), in order to examine specific cell populations that develop during proliferation (as described in materials/methods section 2.5). After being labeled with CFSE, a portion of cells were encapsulated while the remaining were cultured normally as free cells.

Proliferation was examined at specific time points by flow cytometry The cells enclosed within capsules were de-capsulated (using the protocol described in materials/methods section 2.4), prior to being run through the flow cytometer


Free cells
Encapsulated cells
Day 0
Day 1
Day 3
Day 5
AR
AK
AI
F
96.72%
2.2%
94.01%
0.2%
92.05%
4.4%
80.65%
1.5%
0.4%
86.55%
6.6%
55.81%
1.4%
9.60%
88.19%
5.6%
59.42%
3.2%
7.2%
calcein

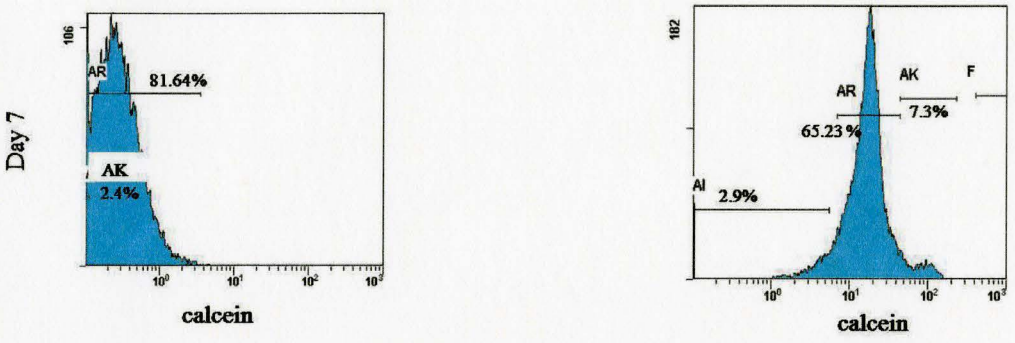


Figure 27 Proliferation profile of CFSE-labelled free and encapsulated G8-cFVIII myoblasts cultured *in vitro*. Cells were assessed on day 0, 1, 3, 5 and 7 by flow cytometry. AR = largest population of live cells that have divided, AK = population of cells that have died, AI = small population of live cells that have undergone successive divisions.

As shown in figure 27, free G8 cells generated the presence of a single main peak (AR) on all days. This result suggests that the large majority of free cells (89.0 ± 5.7 %) divided in synchrony, with only a small proportion of cells (4.2 ± 1.9%) that appeared dead (AK). In contrasts, the cells enclosed in APA capsules showed the presence of three distinct populations: AR, AK, and AI. The majority of encapsulated cells also divided in synchrony as indicated by the dominant AR population. However, as the week progressed, the encapsulated cell distribution began to change. The cell percentage in the AR peak began to decrease, while the dead cell population (AK), which was virtually close to zero on day 1 and day 0, began to grow and stabilize from day 3 onwards. There was also a very small population of encapsulated cells that appeared to have undergone

multiple divisions (AI). The rate of cell proliferation between free and encapsulated cells was compared by observing the change in position over time, of the main peak (AR) along the x-axis. The AR peak of free cells decreased in intensity at faster rate (corresponding to an increase in cell division) than the AR peak of encapsulated cells. By day 7, the free cell peak had approached the lowest end of the intensity scale ($< 10^0$), while the position of the encapsulated cell peak remained virtually stationary (between 10^1 to 10^2) from day 3 onwards; reflecting the decrease in rate of proliferation of encapsulated cells verses non-encapsulated cells.

3.4.2. Proliferation prolife *in vivo*: encapsulated G8-cFVIII myoblasts retrieved from C57BL/6 mouse

The CFSE-labeling of encapsulated cells *in vitro* revealed that their proliferation rates and population distributions were very different from that of free cells. Thus, the affect of the *in vivo* environment on the proliferation of encapsulated cells was investigaed. To our knowledge, the proliferation profile of encapsulated cells implanted into an animal model has never been studied. Thus, a preliminary experiment whereby we implanted CFSE-labeled encapsulated myoblasts secreting cFVIII into a C57BL/6 mouse was performed, and its proliferation profile on day 0 before implantation and on days 7 and 11 after implantation was examined. Non-implanted encapsulated cells were also examined on the same days as a control.

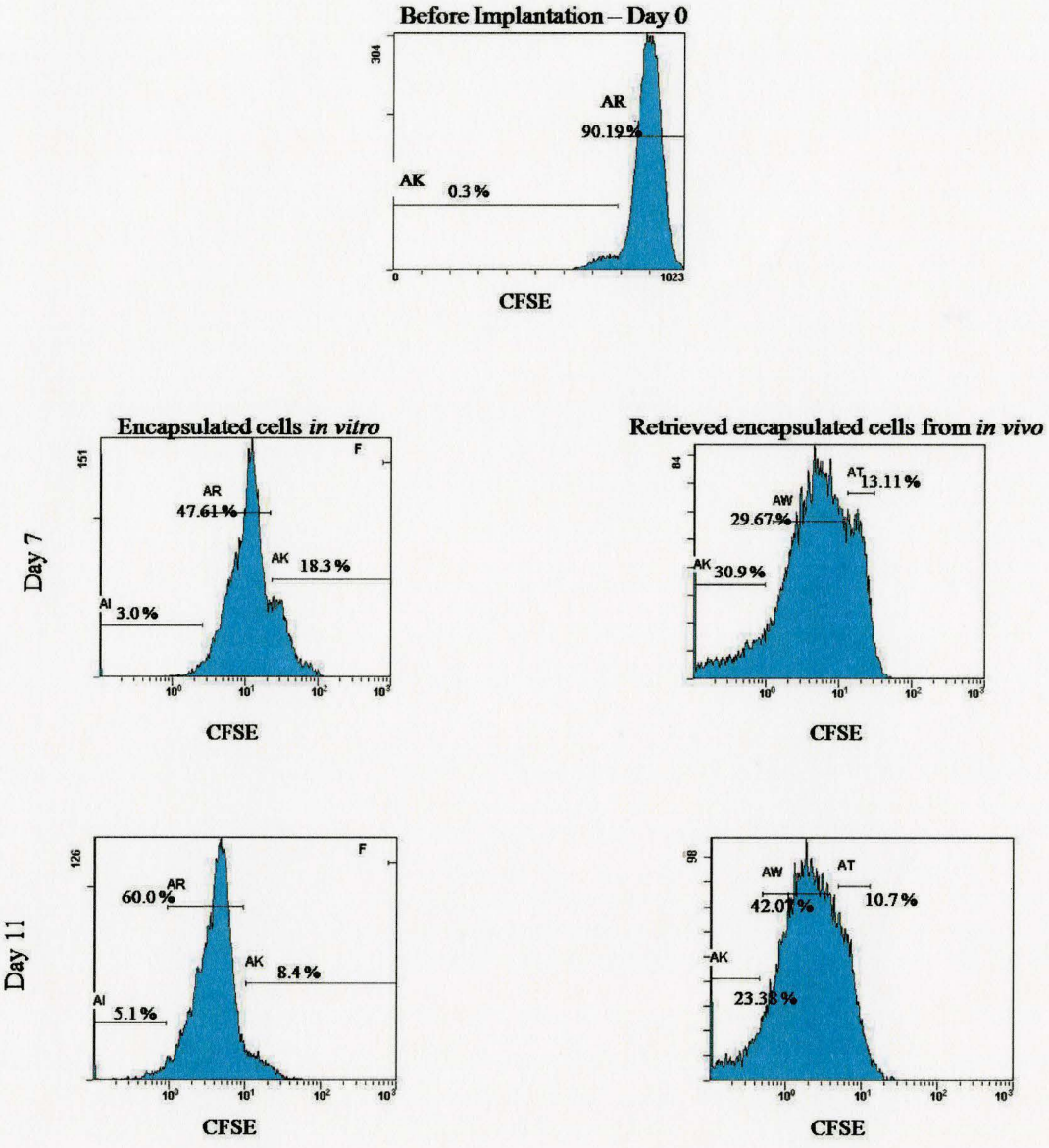


Figure 28 Proliferation profiles of CFSE-labelled non-implanted and implanted encapsulated G8-cFVIII myoblasts. Implanted capsules were retrieved on day 7. Encapsulated cells kept *in vitro* and retrieved encapsulated cells were assessed on day 0, 7, and 11 by flow cytometry. AR = largest population of live cells that have divided *in vitro*. AK = population of cells that have died. AI = small population of live cells that

have undergone successive divisions *in vitro*. AT & AW = largest population of live cells that have divided *in vivo*.

As expected, the non-implanted encapsulated cells produced a proliferation profile similar to the encapsulated cell profile we observed in our *in vitro* experiment from section 3.4.1, showing the presence of the same three distinct populations; one large peak encompassing the majority of cells (AR), a small population of cells that had proliferated multiple times (AI), and a subset of cells that had appeared to be dead (AK) (Figure 28). In contrast, encapsulated cells that had been implanted into mice showed a very different profile after 7 days. There appeared to be the presence of two very large cell populations (AT & AW), proliferating at different rates as indicated by the position of their peaks on the intensity scale (x-axis). When isolated on the forward scatter/side-scatter (FS/SS) flow cytometry plot (not shown), it was noted that these live proliferating cells were distributed over a broad range of sizes. The dead cell population (AK) of the implanted encapsulated cells was also greater than that of the non-implanted encapsulated cells.

3.4.3. Summary of *in vitro* and *in vivo* proliferation experiments

Cellular proliferation is an important characteristic of normal cell behaviour. It relies on a normal cells response to growth factors, signaling molecules and transcription factors. An enclosed environment, much like that of APA microcapsules, may affect normal cellular processes and thus their proliferative capacity [108]. Though it is known that myoblasts limit their proliferation within capsules by differentiating into myotubes, the exact proliferation profile of encapsulated cells has never been examined. Thus, a

flow cytometry based method of measuring free (non-encapsulated) cell proliferation was adapted, so that it could be applied to measuring encapsulated cell proliferation. The proliferation profile of free cells and encapsulated cells *in vitro* was studied, as well as encapsulated cells *in vivo.* It was found that the large majority of free G8 cells cultured *in vitro* were capable of continuous and synchronous cell division, as indicated by a single peak that decreased in CFSE intensity over the week. In contrast, encapsulated G8 cell proliferation slowed down only three days after encapsulation, as the intensity of the main peak remained relatively unchanged from this day onwards. Only a small proportion of encapsulated cells were able to undergo successive divisions over the course of the week. Encapsulation of cells also resulted in the greater accumulation of dead cells. The *in vivo* experiment revealed a very different proliferation profile for the encapsulated cells. There appeared to be two large populations of cells undergoing cell division instead of one main population as was observed *in vitro.*

The CFSE division tracking dye has been commonly used for estimating lymphocyte proliferation and death rates, *in vitro* and *in vivo* [109, 110]. It has been used to quantify the number of divisions immune cells (naïve and memory cells, antigen-specific B and T lymphocytes, etc) have undergone, with and without antigenic simulation [111]. Several mathematical models have been employed for analyzing the CFSE data in terms of the time to first division, the fraction of cells recruited into division, the cell cycle time etc [112]. On a practical level, knowledge of these rates can be important for the clinical assessment of diseases that demonstrate unregulated cell

population growth such as neoplasias. Along with studying lymphocyte dynamics, CSFE tracking has also been used in oncology, stem cell research, and to study the kinetic of bacterial division [113-117]. CFSE labelling of encapsulated cells has never been performed. Our preliminary study of *in vitro* and *in vivo* proliferation rates with encapsulated myoblasts has given us a better understanding of how the capsular environment might affect cell division. The approach of encapsulating genetically modified cells secreting a therapeutic product is being widely explored in gene therapy and cell-based enzyme replacement strategies. Our proliferation studies have established a successful protocol by which encapsulated cell division cycles can be studied and may prompt other gene therapy groups to investigate the behaviour of their specific cell line within capsules; as different cell lines may exhibit different proliferation patterns. The results of our proliferation experiments may be specific to cells secreting the FVIII antigen. It would be of interest to look at encapsulated cells secreting FIX to reveal whether the choice of antigen has an effect on the rate of proliferation. Thus, it is important that additional cell characteristics are studied, such as proliferation in detail, as it could provide additional insight into how the cells' ability for secreting the therapeutic product can be improved.

4. CONCLUSIONS AND FUTURE WORK

4.1. Conclusions

As a result of the examination of cells enclosed in alginate-poly-L-lysine-alginate microspheres performed in the current work, the following conclusions can be drawn.

- The development of a unique flow cytometry protocol for measuring encapsulated cell viability, established a trypsin/EDTA strategy for dissolving the alginate and poly-L-lysine capsule membrane. This method allowed for the release of enclosed cells in solution for subsequent labeling with the fluorescent marker calcein, without adversely affecting cell viability.

- Flow cytometric analysis also allowed for granularity (internal cell composition) measurement of encapsulated cells based on the degree of side-scatter observed on the plot. Thus, instead of solely measuring live and death cell count as limited by other viability assays, this method of viability calculation can also assess the health status of cells based on the change in cell morphology (side-scatter data). This additional piece of analysis may provide insight into the changes in cell secretion as a result of change in encapsulated cell morphology over time.

- Flow cytometry analysis has several advantages over the trypan blue exclusion method including, analysis for a high throughput of cells (thousands per second), several parameters for analysis (intensity, size, and health), and was an objective

assay for viability calculation verses trypan blue which was subjective toward the examiner.

- Encapsulated G8 and BOEC myoblasts maintain a high viability *in vitro* for at least one week. Both cell lines are capable of secreting increasing levels of cFVIII from the capsules at a rate similar to that of hFVIII secretion from encapsulated C2C12 cells [7]

- Implantation of encapsulated G8 myoblasts secreting cFVIII into normal C57BL/6 mice resulted in a statistically significant increase in cFVIII levels on day 1 compared to pre-implantation levels ($p < 0.001$). Levels decreased thereafter and remained low for the remainder of the time, before eventually disappearing on day 35. Accordingly, the viability of encapsulated G8 myoblasts upon retrieval from normal mice was very low on day 0 (30.2 ± 6.6 %), but steadily improved after being cultured *in vitro.* By day 7, viability had approached pre-implantation levels at ~53%.

- Implantation of encapsulated G8 myoblasts secreting cFVIII into hemophilia A FVIII deficient K/O mice delivered therapeutic levels of cFVIII on day 1 which persisted until day 7 before declining. This was a slight improvement over results presented by Garcia *et al* (2002), whose hFVIII levels declined after day 1 and had completely disappeared by day 7. FVIII levels on day 1 in this study were comparable at ~18% to their ~20% of normal levels. Moreover, our observed

decrease on day 14 was only transient as low cFVIII levels returned on day 28 and 35.

- The cFVIII activity in the plasma of treated mice was confirmed by aPTT assay. The increased activity also correlated with the days on which cFVIII antigen concentration was highest.

- Treated mice had high titre of antibodies to cFVIII, in agreement with the findings of Garcia *et al* (2002). The presence of cFVIII activity as indicated by the aPTT assay, may suggest that the antibodies detected in our treated mice were non-neutralizing. However, a Bethesda assay is required to confirm the absence of inhibitors.

- The immediate decline in hFVIII day 1 after implantation, in the work of Garcia *et al* (2002), was attributed to transcriptional repression as measured by reduction in FVIII mRNA from the encapsulated C2C12 cells. The results of the current work do not suggest transcriptional repression to be the *main* reason for decline, as cFVIII levels persisted until day 7, and returned once again by week 5. Moreover, the day 7 therapeutic levels provided protection against blood loss following the tail-clip assay, as confirmed by the unchanged hematocrit levels in treated mice, and the significantly decreased hematocrit levels in the untreated mice.

- Whether encapsulated G8 myoblasts secreting FVIII suffer an inhibition of expression and hence how it compares with retrovirus-driven FVIII expression has yet to be investigated.

- CFSE-labeling of encapsulated G8 myoblasts secreting cFVIII revealed their decreased rate of proliferation compared to free cells. The encapsulated cells also showed a larger dead cell population then free cells. Therefore, encapsulation conditions for G8 myoblasts secreting FVIII must be optimized.

4.2. Future Directions

- The *in vitro* studies using BOECs showed that this cell line was able to secrete increasing concentration of cFVIII from the capsules while maintaining a high viability The next step would be to implant encapsulated BOECs into hemophilic mouse mice and assess its cFVIII delivery *in vivo.*

- Our aPTT data confirmed the presence of cFVIII activity in the treated hemophilic mice at the end of the experiment. To support this data, a Bethesda assay must be carried out to determine whether the anti-cFVIII antibodies detected have an inhibitory affect on cFVIII.

- The results from implanting hemophilic mice with capsules, suggested that the disappearance of the cFVIII activity was transient, and was capable of returning at a later time point. Thus, a long-term experiment should be carried out to establish

whether encapsulated G8 myoblasts are capable of sustained cFVIII secretion over time.

- Along with aPTT, the biological activity on day 7 was also confirmed by the tail clip assay performed on hemophilic mice which were implanted with capsules secreting cFVIII. These mice were protected against blood loss, showing no difference in hematocrit levels from normal mice. The return of cFVIII activity on days 28 and 35 would lead us to perform the tail-clip assay at a later day and evaluate the long-term protection of cFVIII.

- Our proliferation studies of encapsulated cells secreting FVIII revealed the decreased proliferation potential of these cells. It would be of interest to study the proliferation profile of FIX secreting cells to determine whether the pattern of growth is antigen and/or cell line specific.

- Finally, our flow cytometric method of analyzing cellular viability and health could be optimized with the use of a cell-surface specific antibody. This would allow for distinguishing between the cell line of interest, and other smaller/larger immune cells, debris, or cell fragments that appear in either Box A or B. A cell specific marker would increase the accuracy of which viability is reported.

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